Transgenic expression of the amino-terminus of the prion protein and assessment of the nasal cavity as an entry site for prions

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde (Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Claire Bridel

aus Genf

Promotionskommitee

Prof. Dr. med. Dr. sc. h.c. Adriano Aguzzi

Prof. Dr. sc. Roger Morris

Prof. Dr. sc. Peter Seeberger

Zürich, 2009

Table of contents

II. PART I

GENERATION OF A TRANSGENIC MOUSE TO STUDY THE ROLE OF THE FLEXIBLE AMINO-TERMINUS IN THE PHYSIOLOGICAL FUNCTION OF THE PRION PROTEIN

III. PART II

ASSESSMENT OF THE NASAL CAVITY AS AN ENTRY SITE FOR PRIONS

Summary

Prion diseases are infectious neurodegenerative conditions affecting humans and a wide variety of animals. The post-translational conversion of host-encoded cellular prion protein (PrP^{C}) into an abnormal aggregation-prone isoform, designated PrP^{Sc}, is a central feature in the pathogenesis of prion diseases.

The neuronal loss and spongiosis occurring in the central nervous system (CNS) of affected individuals may result from a toxic gain-of-function of aggregated PrP^{Sc} species. However, the contribution of a loss-of-function or dysfunction of PrP^{C} in the pathogenesis of the disease cannot be excluded. Therefore, gaining insight into the yet unclear physiological function of PrP^{C} is of crucial importance for the understanding of prion pathologies.

Transgenic mice expressing amino-truncated versions of the prion protein ($PrP_{\Delta N}$) suffer from spontaneous neurodegeneration. Coexpression of wild-type PrP^{C} rescues the phenotype, suggesting the amino-terminus is crucial for the physiological function of PrP^{C}. In the first part of my thesis, I examined whether the amino-terminus was not only necessary but also sufficient to convey the function of the full-length protein. To do so, I generated a transgenic mouse expressing a redacted version of PrP^{C} consisting solely of its amino-terminus ($PrP_{\Delta 141-231}$). However, because most of the structured domain of the full-length protein is deleted, the resulting protein is unstable and the expression level low. This limits the conclusions that can be drawn from the crossing experiments which show absence of rescue of the phenotype elicited by $PrP_{\Delta N}$ upon $PrP_{\Delta 141-231}$ coexpression.

The mechanisms of transmission of prion diseases among animals are unknown. Hypotheses include oral and cutaneous portals of entry. The identification of novel infection routes is of interest since it may lead to reevaluate tissues and organs otherwise though to be prion free. In the second part of my thesis, I assessed the nasal cavity as a potential entry site for prions. Given the proximity of the nasal cavity with the central nervous system, I hypothesized that prion transmission upon nasal inoculation occurs in the absence of a replicative step in the lympho-reticular system. In a first set of experiments, I show that the nasal pathway is an entry site for prions. In a second, ongoing set of experiments, we examine the requirement of the lympho-reticular system

for prion transmission from the nasal cavity by using mouse models with various degrees of immunodeficiency.

Zusammenfassung

Prionenerkrankungen sind infektiöse neurodegenerative Krankheiten, die Menschen und zahlreiche verschiedene Tierarten befallen können. Dabei ist die post-translationale Konversion des zelleigenen Prion-Proteins (PrP^{C}) zu einer aggregationsanfälligen Isoform, die PrP^{Sc} genannt wird, ein typisches Kennzeichen für Prionenerkrankungen.
Die bei diesen Erkrankungen auftretenden Spongiosen im zentralen Nervensystem (ZNS) könnten von einem toxisch wirkenden gain-of-function Effekt des aggregierten PrP^{Sc} verursacht werden. Allerdings kann auch ein loss-of-function Effekt oder eine Fehlfunktion von PrP^{C} als Ursache für die pathologischen Veränderungen bei diesen Erkrankungen nicht ausgeschlossen werden. Deswegen ist die Aufklärung der immer noch unklaren physiologischen Funktion von PrP^{C} entscheidend für das Verständnis von Prionenerkrankungen.
Transgene Mäuse, die eine amino-terminal verkürzte Version des Prionproteins ($PrP_{\Delta N}$) exprimieren entwickeln eine spontane neurodegenerative Erkrankung. Dieser Phänotyp kann durch Co-expression von Wildtyp-PrP^{C} rückgängig gemacht werden, was darauf hinweist, dass der Aminoterminus wichtig für die physiologische Funktion von PrP^{C} ist.
Im ersten Teil meiner Doktorarbeit untersuchte ich, ob der Aminoterminus nicht nur notwendig ist, sondern auch ausreicht um die Funktion des vollständigen Proteins zu erfüllen. Um das zu erreichen, stellte ich transgene Mäuse her, die eine modifizierte Version von PrP^{C}, die nur aus dem Aminoterminus besteht ($PrP_{\Delta 141-231}$), exprimieren.
Da der grösste Teil der strukturierten Domäne des vollständigen Proteins fehlt, ist das resultierende Protein instabil und schwach exprimiert. Deswegen können aus den Kreuzungsexperimenten, die zeigen, dass die Co-expression von $PrP_{\Delta 141-231}$ mit $PrP_{\Delta N}$ den PrP ΔN-Phänotyp nicht rückgängig macht, nur begrenzt Schlüsse gezogen werden.
Die Mechanismen der Prionenübertragung zwischen Tieren sind unbekannt. Orale oder kutane Eintrittswege werden vermutet. Die Identifikation neuer Infektionswege ist von Bedeutung, da dies zur Re-evaluation von Geweben und Organen führen könnte, die bislang als prionenfrei galten. Im zweiten Teil meiner Doktorarbeit, untersuchte ich, ob die Nasenhöhle eine mögliche Eintrittstelle für Prionen sein könnte. Aufgrund der Nähe der Nasenhöhle zum ZNS, nahm ich an, dass Prionenübertragung nach nasaler

Inokulation ohne einen Replikationsschritt im lympho-retikuären System auskommt. Meine ersten Experimente zeigen, dass die Nasenhöhle eine Eintrittstelle für Prionen ist. In den darauffolgenden Experimenten, die noch nicht abgeschlossen sind, untersuchen wir anhand von Mausmodellen mit unterschiedlichen Graden von Immundefizienz, ob das lympho-retikuläre System notwendig für die Prionentransmission von der Nasenhöhle zum ZNS ist.

Abbreviations

AA	amino acid
bp	base pair
cDNA	complementary DNA
CJD	Creutzfeldt-Jakob disease
fCJD	familial Creutzfeldt-Jakob disease
sCJD	sporadic Creutzfeldt-Jakob disease
vCJD	variant Creutzfeldt-Jakob disease
CNS	central nervous system
CWD	chronic wasting disease
DNA	deoxyribonucleic acid
dpi	days post inoculation
Dpl	Doppel
ELISA	enzyme-linked immunoassay
FDC	follicular dendritic cell
FFI	familial fatal insomnia
GAPDH	glyceralaldehyde 3-phospate deghydogenase
GFAP	glial acidic fibrillary protein
GPI	glycosylphosphatidylinositol
GFAP	glial acidic fibrillary protein
GSS	Gerstmann-Sträussler-Scheinker disease
H-E	hematoxylin and eosin
HRP	horseradish peroxidise
i.c.	intracerebral
i.p.	intraperitoneal
kD	kilo Dalton
LRS	lympho-reticular system
LT	lymphotoxin
ORF	open reading frame

PK	proteinase K
PrP^{C}	cellular prion protein
PrP^{Sc}	abnormal, disease associated isoform of the prion protein
PrP	refers to both PrP^{C} and PrP^{Sc}
$PrP_{\Delta N}$	in this work, designates amino-terminally truncated PrP variants with deletions extending into the hydrophobic core
PrP_{R}	putative receptor of PrP^{C}
Prnd	murine Dpl gene
Prnp	murine PrP^{c} gene
PRNP	human PrP^{c} gene
$Prnp^{0/0}$	*Prnp* knock-out mouse
RML	Rocky mountain laboratory (mouse adapted sheep prion strain)
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
TME	transmissible mink encephalopathy
TNF	tumor necrosis factor
TSEs	transmissible spongiform encephalopathies

I. Introduction

1. Prion diseases

1.1 Definition of prion diseases

Prion diseases are rare fatal neurodegenerative disorders affecting both humans and animals. They bear morphological and pathophysiological similarities with other neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, but are unique in that they are transmissible. In reference to their infectious nature and the typical spongiform degeneration that occurs in the central nervous system (CNS) of affected individuals (figure 1), prion diseases are also referred to as transmissible spongiform encephalopathies (TSEs).

The nature of the infectious agent causing TSEs, called the prion, has not been determined with certainty. The most widely accepted hypothesis suggests that PrP^{Sc}, a misfolded β-sheet rich version of the normal host prion protein PrP^{C} is a critical, if not the only component of the prion (Prusiner 1982) (Weissmann 1991).

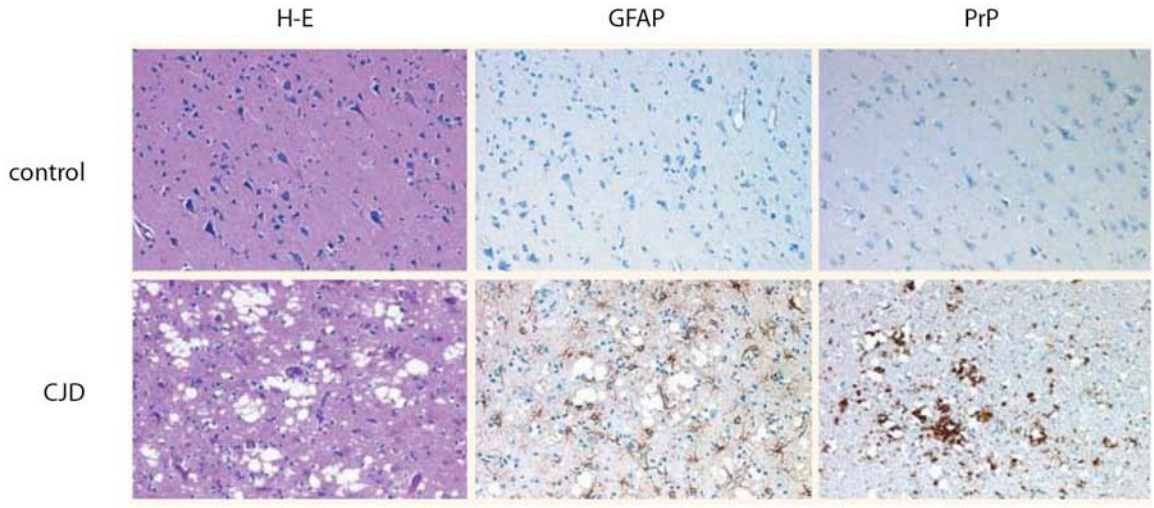


Figure 1

Neuropathological features of transmissible spongiform encephalopathies. Histological and immunohistochemical analysis of frontal cortex samples from the brain of a patient who died of non-cerebral causes (upper row) and of a patient with Creutzfeldt-Jakob disease (CJD; lower row). Brain sections were stained with haematoxylin-eosin (H-E, left panels), with antibodies against glial fibrillary acidic protein (GFAP, middle panels) and with antibodies against the prion protein (PrP, right panels). Neuronal loss and prominent spongiosis are visible in the H-E stain. Strong proliferation of reactive

astrocytes (gliosis) and perivacuolar prion protein deposits are detectable in the GFAP and PrP immunostains of the CJD brain samples. From Aguzzi e*t al.*, Nat Rev Neurosci, 2001.

1.2 A historical perspective

The first record of prion diseases dates back to mid-18th-century England, when a spreading disease in sheep, then one of the most widely exchanged goods, alarms the British parliament (Brown and Bradley 1998). Affected animals typically develop tremor, ataxia and pruritus, leading them to scrape or bite the affected areas. The name of the disease, scrapie, derives from this behaviour (Lampert, Gajdusek et al. 1972). The transmissible nature of the disease is recognized very early, but is first proven in 1936 by Cuillé and Chelle who successfully transmit scrapie to two healthy sheep by intraocular inoculation of CNS tissue derived from an affected animal (Cuillé 1936). Ten years later, the infectious property of the scrapie agent is involuntarily confirmed in England following an outbreak of scrapie among sheep immunized against louping-ill with a vaccine manufactured with CNS and spleen tissue of scrapie-affected animals (Gordon 1946). However, the aetiology of the disease remains a mystery, and speculations include a parasitic or viral origin (Plummer 1946).

In the 1950's, the Fore people, inhabitants of Papua-New Guinea, come to the attention of the scientific community. Australian anthropologists Ronald and Catherine Berndt report a unique neurological condition, locally called kuru, restricted to the Fore people and reaching epidemic proportions (Berndt 1954). The aetiology of kuru is obscure, but seems to be linked to ritualistic endocannibalism (Goldfarb 2002). The interest of American paediatrician and virologist Gajdusek is tickled (Gajdusek and Zigas 1957), and he conducts a series of inoculation experiments leading to the proof of transmissibility of kuru to chimpanzees (Gajdusek, Gibbs et al. 1966). In 1959, neuropathologist Klatzo points out the similarity between kuru and another human neurological condition of unknown aetiology, described in the early 1920's by two German neurologists Creutzfeldt and Jacob (Creutzfeldt 1920) (Jakob 1921) (Klatzo, Gajdusek et al. 1959). Similarly to kuru, Creutzfeldt-Jakob disease (CJD) is shown to be transmissible to chimpanzees (Gibbs, Gajdusek et al. 1968). The same year, the American veterinary neuropathologist Hadlow suggest an analogy between kuru and the sheep disease scrapie, based on the striking similarities of the neuropathologic findings (Hadlow 1995). Scrapie, kuru and CJD are believed to share the same aetiology

(Lampert, Gajdusek et al. 1972), but the nature of the infectious agent is debated. Until the 1980's, the scientific community favours a slow viral origin, which explains both the infectious nature and the unusually long incubation period of the diseases (Lampert, Gajdusek et al. 1972) (Gajdusek 1977).

The first step towards discovering what is now widely accepted as the causative agent of prion diseases is made by British radiopathologist Alper. In 1966, on the basis of experiments showing the absence of inactivation of the scrapie infectious agent following exposure to ultra-violet (UV) light (Alper, Haig et al. 1966), she suggests that the scrapie agent has the unique property of being able to replicate in the absence of nucleic acids (Alper, Cramp et al. 1967) (Alper, Haig et al. 1978). A year later, this revolutionary idea is supported by a mathematical model proposed by Griffith, who suggests that under certain circumstances, proteins are able to self-replicate (Griffith 1967).

Fifteen years later, American neurologist and biochemist Prusiner demonstrates that the scrapie agent is resistant to procedures inactivating nucleic acids, but is sensitive to treatments denaturing or hydrolysing proteins (Prusiner 1982). Based on these findings, he proposes that the scrapie agent, and by extension the causative agent of all other prion diseases, is a proteinaceous molecule devoid of nucleic acids. This 'protein-only hypothesis' further suggests that an abnormal isoform of the host-encoded cellular prion protein (PrP^{C}) is the principal and possibly the only constituent of the transmissible agent (Bolton, McKinley et al. 1982) (Prusiner 1982). This abnormal isoform, called PrP^{Sc}, induces a conformational change in PrP^{C}, turning it into a like of itself (Weissmann 1991).

The demonstration that *Prnp* knock-out mice are resistant to prion infection (Bueler, Aguzzi et al. 1993) and that prion diseases can be caused by a mutation in the host prion protein gene support the protein only hypothesis which is now widely accepted among the scientific community.

1.3 Animal prion diseases

Naturally occurring animal prion diseases include sheep scrapie, the prototypical animal prion disease, bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD) in deer and elk and transmissible mink encephalopathy (TME).

Scrapie is an enzootic disease of sheep first reported in the 18th century, more prevalent in the northern than in the southern hemisphere. Epidemiological studies on transmissibility of scrapie to humans have never shown a causal effect (Chatelain, Cathala et al. 1981), and scrapie is not considered a zoonosis (Smith and Bradley 2003).

Unlike scrapie, the discovery of the other naturally occurring animal TSEs is more recent. The first case of BSE, also called “mad cow disease” is thought to have occurred in 1985 in the United Kingdom (Bradley 2001), although a French report from the late 19th century describes a case clinically similar to BSE in an ox (Sarradet 1883). The origin of this first case is unknown, and speculations include transmission of scrapie to cattle or a rare sporadic occurrence of the disease in cattle (Smith and Bradley 2003). The epidemic spread of the disease in the UK during the late 1980’s to mid 1990’s is attributed to transmission of cattle-adapted scrapie or BSE to cattle via contaminated feed prepared from rendered carcasses, called meat and bone meal (MBM) (Horn 2001). The epidemic, which peaked in 1993, was controlled by banning ruminant offal as raw material for the preparation of dietary supplements destined for feeding to cattle or other ruminants (Sellier 2003). BSE was subsequently detected in other European countries, although to a lower extent than in the UK. It is hypothesized that these cases result from exportation of cattle, cattle products or meat and bone meal (MBM) from the UK (Smith and Bradley 2003). The higher incidence observed in the UK is suggested to be due to several circumstances, including a high ratio of sheep to cattle, a high enzootic prevalence of scrapie in sheep, the intensive feeding of MBM to dairy cattle and the changes in rendering practices (Prusiner 2004).

CWD is a TSE of captive and free-ranging mule deer, white-tailed deer and Rocky-Mountain elk (Williams and Young 1992). The first cases of CWD are recognized among captive mule deer in the late 1960’s in Colorado. However the prion aetiology of the disease is first appreciated in 1977 (Williams and Young 1980). In contrast to scrapie and BSE, where the clinical manifestations of the disease are predominantly neurological, loss of body condition and emaciation are the prominent features of CWD

(Sigurdson 2008). Since the 1960's, CWD has widely spread in North America following natural movement of animals along migration corridors.

TME is a rare sporadic disease of ranched mink, first recognized in 1947 in Wisconsin and Minnesota (Marsh and Hadlow 1992). Outbreaks in the 1960's are epidemiologically associated with consumption of feed of ovine or bovine origin (Hartsough and Burger 1965), and an unrecognized case of BSE has been suggested as the cause of TME (Marsh, Bessen et al. 1991).

1.4 Human prion diseases

Human prion diseases are unique in that the disease process can be triggered through an inherited germline mutation in the prion protein gene (*PRNP*), through infection following exposure to PrP^{Sc} or sporadically through the rare spontaneous conversion of PrP^{C} into PrP^{Sc}.

Sporadic CJD (sCJD) is the most frequent type of human prion diseases, accounting for 85% of all cases. It is rare, with an incidence of 1-2 cases per million population per year across the world (Brown, Cathala et al. 1987). sCJD has no obvious infectious or genetic aetiology, and is postulated to be caused by a somatic mutation in the human prion gene *PRNP* or alternatively by the rare spontaneous conversion of PrP^{C} into PrP^{Sc} (Collinge 1997). The transmissibility of sCJD to non human primates was first demonstrated in 1968 (Gibbs, Gajdusek et al. 1968). Clinically, sCJD is a rapidly progressive multifocal dementia, usually accompanied by myoclonus. The peak of onset is between 60-65 year, and death occurs within 2-3 months (Wadsworth, Hill et al. 2003). Neuropathological findings include the classical triad of neuronal loss, astrogliosis and spongiform change (figure 1). PrP amyloid plaques are usually not present (Budka, Aguzzi et al. 1995).

About 15% of human prion diseases are familial (inherited), associated with a mutation in *PRNP*. To date, more than 50 mutations have been identified and linked to a familial prion diseases (Prusiner 2004). Familial prion diseases are further sub-classified according to the clinical manifestations of the disease, although a high degree of phenotypic overlap exists between individuals with different mutations (Collinge, Brown et al. 1992). The most frequent familial prion disease is familial CJD (fCJD), and over 20 mutations co segregate with this disease. Clinically, fCJD is very heterogeneous but classically resembles sCJD (Parchi, Castellani et al. 1996). Fatal Familial insomnia (FFI) is most frequently associated with a D178N missense mutation. Progressive untreatable insomnia, dysautonomia, dementia and selective thalamic degeneration classically characterize this disease (Medori, Tritschler et al. 1992). Gerstmann-Sträussler-Scheinker disease (GSS) is most commonly associated with a P101L substitution. Chronic cerebellar ataxia with pyramidal features are the initial signs of the diseases, dementia occurring much later in the clinical course. The duration of the disease is typically longer than in sCJD (Gerstmann 1928) (Collinge 1997). The mechanisms by

which these mutations trigger the disease remain to be uncovered, but it is speculated that the prion protein bearing a pathogenic mutation has a high propensity to convert spontaneously to the infectious isoform (Prusiner 1989). Experimental transmission of fCJD (Roos, Gajdusek et al. 1973) and GSS (masters 1981) to non-human primates and FFI to mice (Tateishi, Brown et al. 1995) proved the infectious nature of these genetic diseases.

Acquired prion diseases are the rarest form of human TSEs. They include prion diseases transmitted by iatrogenic contact with infected human material (such as dura mater or growth hormone derived from human cadavers) or by dietary exposure to prions, such as kuru or variant CJD (vCJD). vCJD was first described in 1996 in the UK (Will, Ironside et al. 1996). Clinically, the initially predominantly psychiatric disturbances, the early onset of disease and the comparatively long clinical course clearly distinguish vCJD from other human prion disease. Neuropathologically, vCJD cases display large numbers of PrP-containing amyloid plaques, a feature that is rarely found in sCJD (Budka, Aguzzi et al. 1995). Based on epidemiological, neuropathological and biochemical findings, vCJD is believed to arise from ingestion of BSE contaminated beef (Collinge and Rossor 1996) (Weissmann and Aguzzi 1997).

In addition to the mutations linked to inheritable prion disease, 16 polymorphisms have been identified in *PRNP*. A coding polymorphism at codon 129 of *PRNP* is a strong susceptibility factor for human prion diseases. About 38% of Europeans are homozygous for the more frequent methionine allele, 51% are heterozygous, and 11% homozygous for valine (Wadsworth et al. 2007). Homozygocity for methionine at codon 129 (M/M) predisposes to sporadic and acquired CJD (Collinge, Palmer et al. 1991) (Palmer, Dryden et al. 1991). The polymorphism also affects the disease course of iatrogenic CJD, with heterozygotes (methionine/valine) displaying a longer incubation time (Huillard d'Aignaux, Costagliola et al. 1999).

1.5 Yeast and filamentous fungi prions

Prions can be broadly defined as infectious proteins that do not require a nucleic acid for infectivity. Such proteins have been identified in yeast and filamentous fungi, and are commonly referred to as yeast prions. Their amino acid sequence does not bear any homology with PrP^{C}, but like mammalian prions, yeast prions consist of altered versions of normal cellular proteins which impose their conformation onto their normal counterpart to propagate. Up to now six yeast prions have been identified, four of which are amyloidogenic and two others consisting of self-activating enzymes (Wickner, Edskes et al. 2007). The amyloidogenic yeast prions bare similarities with PrP^{Sc} in that they aggregate and form amyloid fibrils. But in contrast to mammalian prions, they are not pathogenic, and protein aggregation is associated with loss of function of the normal protein.

2. The cellular prion protein and its abnormal isoform

2.1. Biosynthesis, trafficking and cleavage of the cellular prion protein

The mouse PrP^C polypeptide consists of 254 amino-acids. A 22 amino-acid signal sequence targets PrP^C to the endoplasmatic reticulum (ER) and after translocation, 23 hydrophobic amino-acids are cleaved off from the carboxy-terminus and replaced by a glycosyl phosphatidyl inositol (GPI) anchor (Stahl, Borchelt et al. 1987), giving rise to a 209 amino acid protein. In the ER and Golgi, the 25 kD protein is facultatively glycosylated at two asparagine residues, giving rise to un-, mono- and diglycosylated PrP^C species (Bolton, Meyer et al. 1985) (Haraguchi, Fisher et al. 1989). The mature protein is transported via secretory granules to the cell membrane, to which it is attached by its GPI anchor. PrP^C is located in cholesterol and sphingolipid rich microdomains of the membrane, called lipid rafts (Vey, Pilkuhn et al. 1996) (Naslavsky, Stein et al. 1997).

The protein constitutively cycles between the plasma membrane and early endosomes. The mechanism of PrP^C internalization is still controversial and both clathrin-dependant endocytosis (Shyng, Heuser et al. 1994) (Taylor, Watt et al. 2005) and a caveola-mediated endocytosis (Vey, Pilkuhn et al. 1996) (Kaneko, Vey et al. 1997) (Marella, Lehmann et al. 2002) (Peters, Mironov et al. 2003) have been reported.

Two posttranslational cleavages occur as part of the normal metabolism of PrP^C (Mange, Beranger et al. 2004) (Hooper 2005). One cleavage takes place within the GPI and releases the polypeptide chain into the extracellular matrix (ECM) (Borchelt, Scott et al. 1990). The second cleavage is proteolytic (α cleavage) and occurs within a segment of 16 hydrophobic amino acids that is conserved in all cloned PrP^C species (Harris, Huber et al. 1993). This cleavage gives rise to a soluble amino-terminal fragment (N1) and a GPI-anchored carboxy-terminal fragment (C1) that can no longer be converted into PrP^{Sc} (Harris, Huber et al. 1993) (Jimenez-Huete, Lievens et al. 1998) (figure 2).

Upon exposure to reactive oxygen species (ROS), PrP^C was shown to be cleaved *in vitro* within or adjacent to the octarepeat region (β-cleavage), to generate N2 and C2 fragments (McMahon, Mange et al. 2001) (figure 2). The C2 fragment was also detected

specifically in the brain of patients suffering from CJD (Chen, Teplow et al. 1995). The significance of both the α and β cleavages of PrP^C remains to be determined.

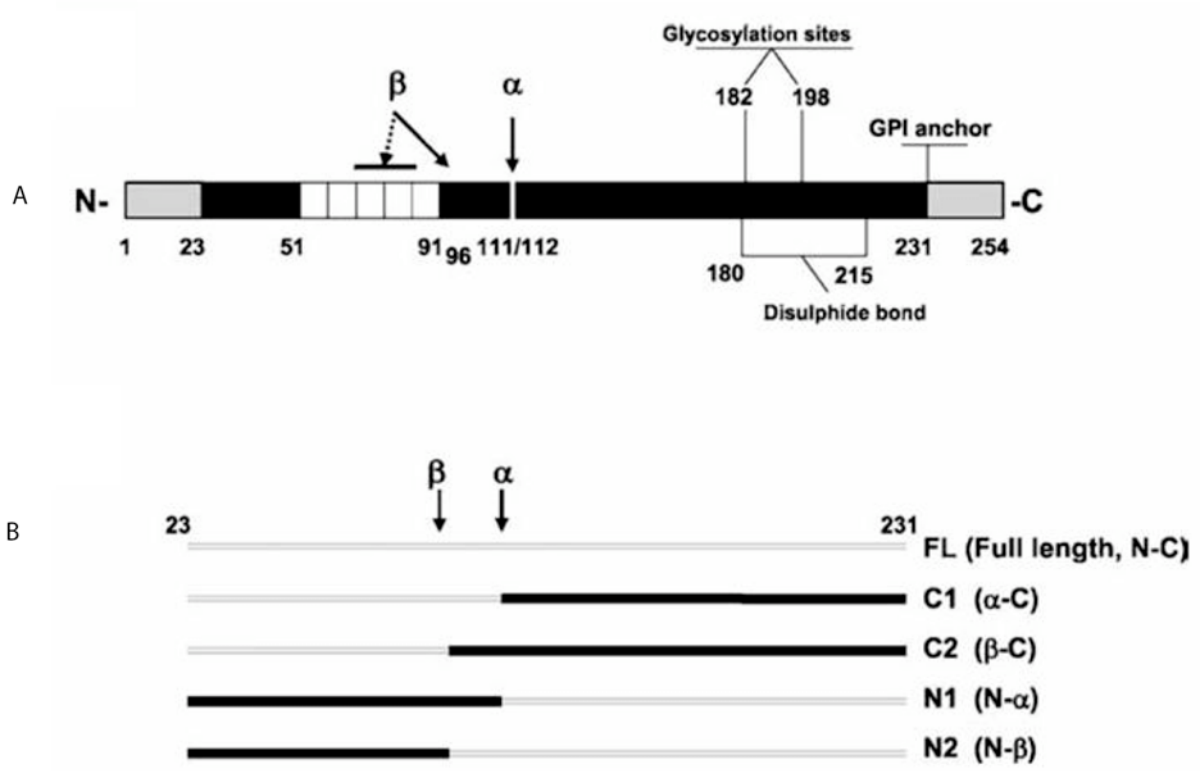


Figure 2

α- and β-cleavage of mouse PrP^C. A Schematic representation of mouse PrP^C. Amino- and carboxy-terminal signal peptides, octarepeat region, N-linked glycosylation sites, disulfide bridge and attachment of the GPI anchor are represented. The α-cleavage site is located at position 111/112; the β-cleavage site is located in the octarepeat region, near histidine position 96. **B** Nomenclature of the PrP^C fragments generated by the α- and β-cleavage. Adapted from Mangé e*t al.*, Biol Cell, 2004.

2.2 Expression pattern of the cellular prion protein

The cellular prion protein is highly conserved among mammals (Rivera-Milla, Stuermer et al. 2003) (Westaway and Carlson 2002) (figure 3) and orthologs have been identified in other vertebrates such as marsupial (Windl, Dempster et al. 1995), chicken (Harris, Lele et al. 1993) and fish (Rivera-Milla, Stuermer et al. 2003). Murine, hamster and human PrP genes were cloned in 1986 (Locht, Chesebro et al. 1986) (Basler, Oesch et al. 1986) (Liao, Lebo et al. 1986). In all known mammalian PrP^C genes, a single open reading frame encodes the protein. Within the genome, the PrP^C gene is present in a single copy. The mRNA is constitutively expressed in the adult organism and developmentally regulated during mouse embryogenesis (Manson, West et al. 1992) (Miele, Alejo Blanco et al. 2003). PrP^C is ubiquitously expressed although most abundantly in the CNS (Ford, Burton et al. 2002) (Bendheim, Brown et al. 1992). Within the CNS, PrP^C is most highly expressed by neurons, although astrocytes and oligodendrocytes also express it (Moser, Colello et al. 1995) (Sales, Hassig et al. 2002).

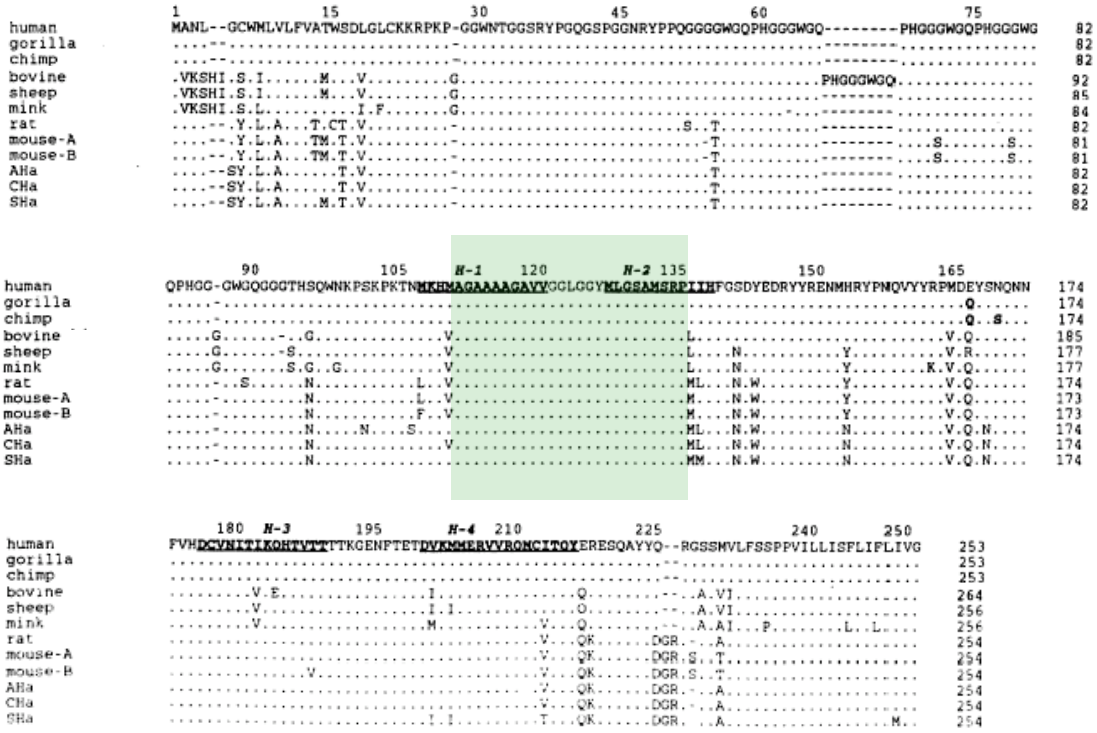


Figure 3

Alignment of deduced amino acid sequences of human, gorilla, chimp, bovine, sheep, mink, rat, mouse and hamster prion proteins. Dots represent amino acids that are identical with the human PrP^{C} sequence. Lines represent amino-acids that do not exist in the respective sequence. The most conserved region of the prion protein is a hydrophobic stretch that spans from amino acid 112 to 136 (human numbering), called the hydrophobic core (HC, in light green). Adapted from Van Rheede *et al.*, Mol Biol Evol, 2003.

2.3 Structure of the cellular prion protein

PrP^C consists of a highly structured carboxy-terminus and a flexible amino-proximal domain. The globular carboxy-terminal domain (mouse amino acid residues 135-231) comprises three α-helices (mouse amino acid residues 141-154, 175-193 and 200-219) and an anti-parallel β-sheet (mouse amino-acid residues 128-131 and 161-164). A disulphide bridge connects helix 2 and 3, and residue 180 and 196 are two facultative N-glycosylation sites (Riek, Hornemann et al. 1996) (Riek, Hornemann et al. 1997).

Knock-in mice carrying point mutations disrupting one or both N-glycosylation sites (N180T, N196T, N180T/N196T) are normal, although complete blockade of glycosylation (N180T/N196T) leads to a predominantly intracellular location of PrP^C (Cancellotti, Wiseman et al. 2005). Transgenic mice overexpressing a deglycosylated isoform of PrP^C are susceptible to scrapie and BSE (Neuendorf, Weber et al. 2004), indicating that glycosylation is dispensable for prion replication.

Four defined regions are recognized within the flexible amino-terminal domain of PrP^C (amino acid residues 23-134). First, a stretch of five positively charged amino acids situated at the very amino-terminus and called charged cluster 1 (CC1, mouse amino acids 23-27), that has been shown to be crucial for PrP^C internalisation (Sunyach, Jen et al. 2003) (Taylor, Watt et al. 2005). Second, a copper binding region consisting of a segment of four (murine PrP^C) or five (human PrP^C) repeats of a sequence of eight amino acids, called the octarepeat region (OR, mouse amino acids 60-91). Transgenic mice expressing PrP^C variants lacking the OR develop normally (Fischer, Rulicke et al. 1996) (Shmerling, Hegyi et al. 1998) (Muramoto, DeArmond et al. 1997) and propagate prions efficiently, indicating that the OR is neither of crucial importance for the physiological function of PrP^C nor for the conversion to PrP^{Sc}. But interestingly, transgenic mice expressing a PrP^C variant with nine supernumerary octapeptide repeats, modelling a fCJD mutation, develop a cerebellar disorder (Chiesa, Drisaldi et al. 2000). The disease is not transmissible, and cannot be rescued by co-expression of the full-length protein (Chiesa, Piccardo et al. 2003). The third domain, called charged cluster 2 (CC2, amino acids 95-110) precedes a highly conserved region, the hydrophobic core (HC, amino acids 111-134) (figure 3). While transgenic mice expressing PrP^C variants with disrupted CC2 regions do not develop a phenotype, disruption of the HC leads to

spontaneous neurodegeneration (Baumann, Tolnay et al. 2007) (Li, Christensen et al. 2007), suggesting an important role for the HC in the physiological function of PrP^C.

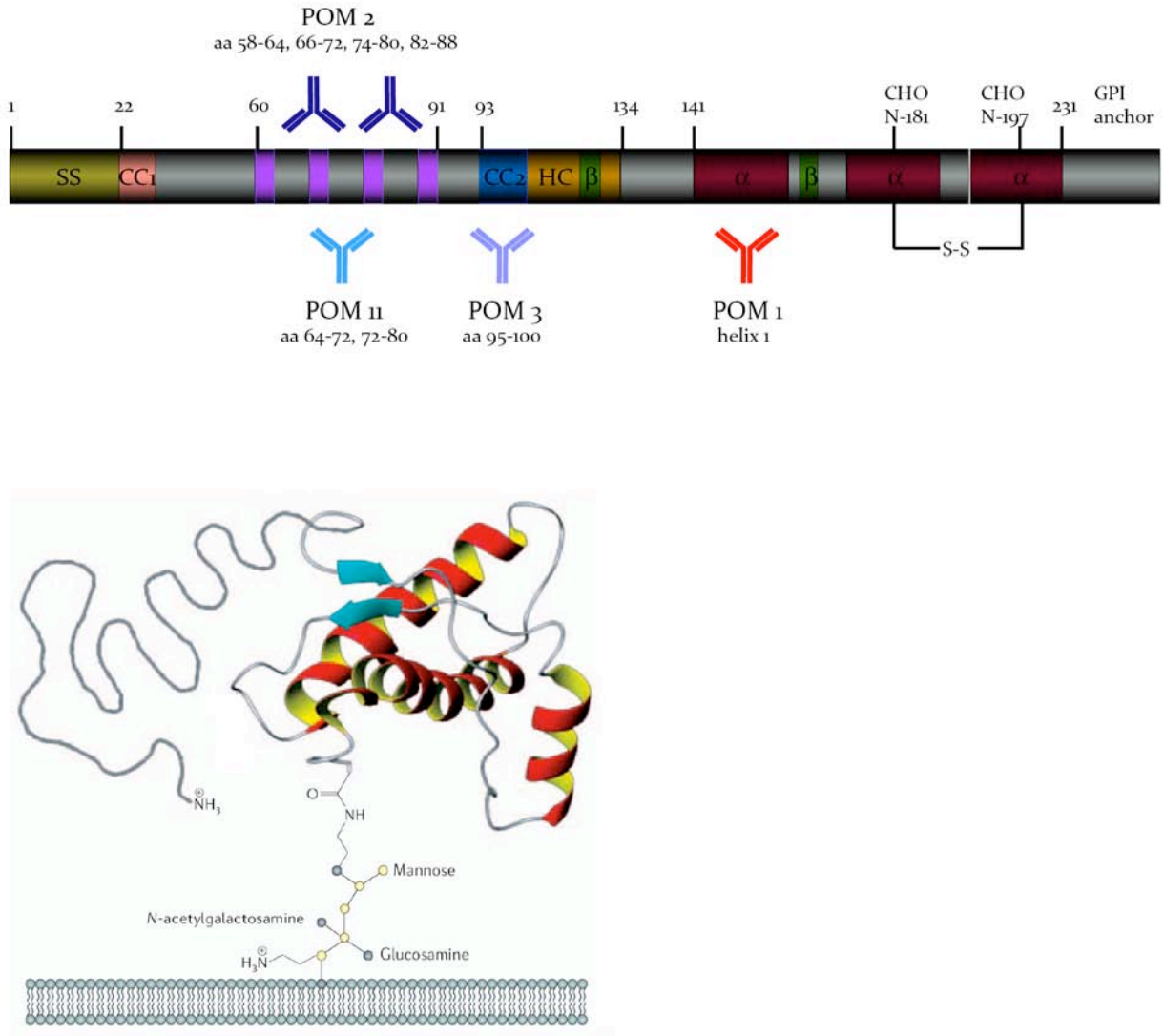


Figure 4

Structural features of the cellular prion protein. A Primary structure of the cellular prion protein including post-translational modifications. The numbers describe the position of the respective amino acids. SS (yellow) indicates the signal sequence; CC1 (pink) represents the charged cluster 1; OR (purple) designates the octarepeat region; CC2 (dark blue) represents the charged cluster 2 and HC (orange) the hydrophobic core. The beta sheets are depicted in green and the alpha helices in red. S-S indicates the disulphide bridge. GPI denotes the membrane anchorage region. **B** Tertiary structure of the cellular prion protein inserted into a lipid bilayer, as deduced from NMR spectroscopy, including the unstructured amino-terminal region depicted in grey and the GPI anchor. Adapted from Aguzzi *et al.,* Nat Rev Microbiol, 2006.

2.4 Physiological function of the cellular prion protein

The role of PrP^C in the pathogenesis of prion diseases is well established (Bueler, Aguzzi et al. 1993), but its physiological function remains unclear. One major draw-back towards understanding the physiological function of PrP^C is the lack of phenotype in the CNS of *Prnp*$^{0/0}$ mice (Bueler, Fischer et al. 1992). Furthermore, mice in which PrP^C is post-natally deleted fail to develop a phenotype, arguing against the existence of other proteins that compensate for essential PrP^C functions during development (Mallucci, Ratte et al. 2002). These findings suggest either redundancy of PrP^C or the absence of a major function of the protein in the CNS. PrP^C being a highly conserved protein most abundantly expressed in the CNS, the latter hypothesis is unlikely.
Many functions are attributed to PrP^C, supported by both *in vitro* and *in vivo* evidence, however a unified view of the function is still missing

PrP^C and apoptosis

PrP^C has been suggested to convey both pro- and anti-apoptotic signals.
In cultured human foetal neurons, PrP^C was found to protect against Bax mediated apoptosis (Bounhar, Zhang et al. 2001) (Roucou, Guo et al. 2003) (Roucou, Giannopoulos et al. 2005), and PrP^C expression was shown to rescue Hpl cells from serum deprivation induced apoptosis (Kuwahara, Takeuchi et al. 1999).
In contrast to these findings, overexpression of PrP^C was shown to sensitize cultured cells to staurosporin induced death and increase caspase 3 activity (Paitel, Alves da Costa et al. 2002), and cross-linking of PrP^C on the neuronal surface was shown to induce apoptosis in hippocampal and cerebellar neurons *in vivo* (Solforosi, Criado et al. 2004).

PrP^C and oxidative stress

Oxidative stress plays a central role in the pathogenesis of neurodegenerative diseases (Halliwell 2006) and PrP^C has been suggested to be involved in cellular protection against oxidative stress.

Cerebellar granular and neocortical neurons isolated from *Prnp*$^{0/0}$ mice were shown to be more susceptible than *Prnp*$^{+/+}$ neurons to treatments with oxidative stress inducing agents (Brown, Schmidt et al. 1997) (Brown, Schulz-Schaeffer et al. 1997) (Buddi, Lin et al. 2002). Also, increased levels of oxidative stress markers were found in brains of *Prnp*$^{0/0}$ mice compared to *Prnp*$^{+/+}$ mice. In addition, brain lesions induced by hypoxia and ischemia are larger in *Prnp*$^{0/0}$ mice than in wild-type mice (McLennan, Brennan et al. 2004). The mechanisms underlying the putative protective effect of PrP^{C} are controversial. It has been suggested that PrP^{C} has a super oxide dismutase (SOD) activity (Brown, Wong et al. 1999), but theses findings could not be confirmed *in vivo* (Hutter, Heppner et al. 2003).

PrP^{C} and copper

PrP^{C} binds copper via the octarepeats of its amino-terminus (Brown, Qin et al. 1997). Copper was shown to stimulate the endocytosis of PrP^{C} (Pauly and Harris 1998) (Perera and Hooper 2001), suggesting that PrP^{C} functions as a receptor for cellular uptake or efflux of copper ions. However, a correlation between PrP^{C} expression and the cellular or tissue content of copper could not be found *in vivo* (Waggoner, Drisaldi et al. 2000).

PrP^{C} as a signalling molecule

Lipid rafts are specialized microdomains of the plasma membrane enriched in cholesterol and sphingolipids. Rafts are thought to play a facilitating role in signal transduction (Tsui-Pierchala, Encinas et al. 2002) . The presence of PrP^{C} in lipid rafts suggests it participates in transmembrane signalling processes. Antibody cross-linking of PrP^{C} on neuroectodermal cell line 1C11 was found to stimulate the activity of non-receptor tyrosine kinase Fyn via interaction of PrP^{C} with raft protein caveolin (Mouillet-Richard, Ermonval et al. 2000), leading to downstream stimulation of NADPH oxidase and extracellular regulated kinases (Erks) as well as production of reactive oxygen species (ROS) (Schneider, Mutel et al. 2003). Also, PrP^{C} was shown to facilitate neurite outgrowth via cis and trans interactions with NCAM to lipid rafts and activation of Fyn kinase (Santuccione, Sytnyk et al. 2005). However, *in vivo* confirmation of these findings is still missing.

2.5 Interaction partners

A common strategy to elucidate the physiological function of a protein is to identify interacting partners. In the case of PrP^C many different interacting partners have been described (Watts and Westaway 2007) (Aguzzi, Baumann et al. 2008). However, the lack of overlap between the putative interacting partners identified in different laboratories and the inability to prove functional relevance of the interactions *in vivo* have undermined the physiological relevance of these findings.

2.6 The disease associated-isoform of the prion protein

This structural transition of PrP^{C} to PrP^{Sc} is accompanied by profound changes in the physicochemical properties of the prion protein. While PrP^{C} is soluble in mild detergents and sensitive to proteinase K (PK), PrP^{Sc} is insoluble, forms aggregates and is partially resistant to PK (Oesch, Westaway et al. 1985) (Meyer, McKinley et al. 1986). PK cleavage of PrP^{Sc} around amino acid residue 90 (McKinley, Meyer et al. 1991) gives rise to a fully infectious prion protein species, named PrP^{27-30} after its migration pattern on a SDS-PAGE (McKinley, Meyer et al. 1991) (figure 5). The insolubility of PrP^{Sc} prevents NMR studies, and first indications of differences in the secondary structure of the two PrP isoforms came from infra-red spectroscopic measurements. While PrP^{C} is dominated by α-helices and has only little β-sheet content, PrP^{Sc} is characterized by similar amounts of α-helices and β-sheets (Caughey, Dong et al. 1991) (Safar, Roller et al. 1993).

In most cases of naturally occurring and experimentally induced prion diseases, a correlation exists between PK resistance and infectivity, and PK resistant material is used as a surrogate marker for infectivity. However, it appears that disease associated isoforms of the prion protein can be PK sensitive (Lasmezas, Deslys et al. 1997) (Hsiao telling1994), and that PK resistant PrP is not always infectious (Chiesa 2000).

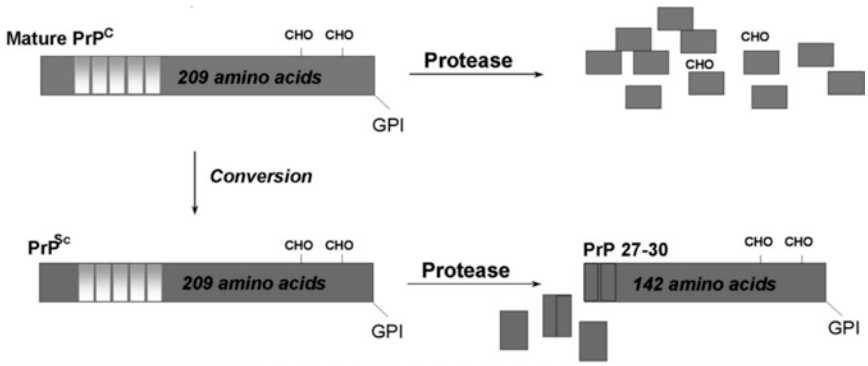


Figure 5

Schematic drawing of PrP^{C} and PrP^{Sc} before and after Proteinase K (PK) digestion. The mature human prion protein contains 209 amino acids. The five light grey boxes indicate the octarepeats of the amino-terminal domain. PrP^{C} and PrP^{Sc} have an identical amino acid sequence and share the same posttranslational modifications, but differ in their secondary and (presumably) tertiary structure. The physiological isoform PrP^{C} is protease-sensitive, while the pathological isoform PrP^{Sc} is partially protease resistant, displaying a protease-resistant core designated PrP^{27-30}. Adapted from Aguzzi e*t al.*, Cell Death Differ, 2000.

3. Mechanisms of prion transmission and neurodegeneration

3.1. Conversion of PrP^{C} into PrP^{Sc}

Mechanisms of conversion

According to the protein-only hypothesis, the disease associated isoform of the prion protein propagates by imposing its conformation onto the normal cellular protein PrP^{C}, turning it into a like of itself (Prusiner 1982). PrP^{Sc} then accumulates in the brain of affected individuals, either as amorphous deposits or as highly structured amyloid (Budka, Aguzzi et al. 1995). Two hypotheses have been formulated to explain the mechanisms leading to the conversion of PrP^{C} into PrP^{Sc} (Weissmann 1999). The "refolding model" (figure 6a) suggests that PrP^{C} can spontaneously refold into PrP^{Sc}, but that this phenomenon is prevented by a high activation energy barrier. In the presence of exogenous PrP^{Sc}, the reaction is facilitated. The "seeding (or nucleation) model" (figure 6b) proposes that PrP^{C} and PrP^{Sc} are in equilibrium. PrP^{Sc} is stabilized when added onto an aggregate of PrP^{Sc} or seed. The initial formation of the seed is a rare event, but once formed, additional PrP^{Sc} molecules can add up onto the seed which grows and eventually breaks, leading to an exponential conversion rate.

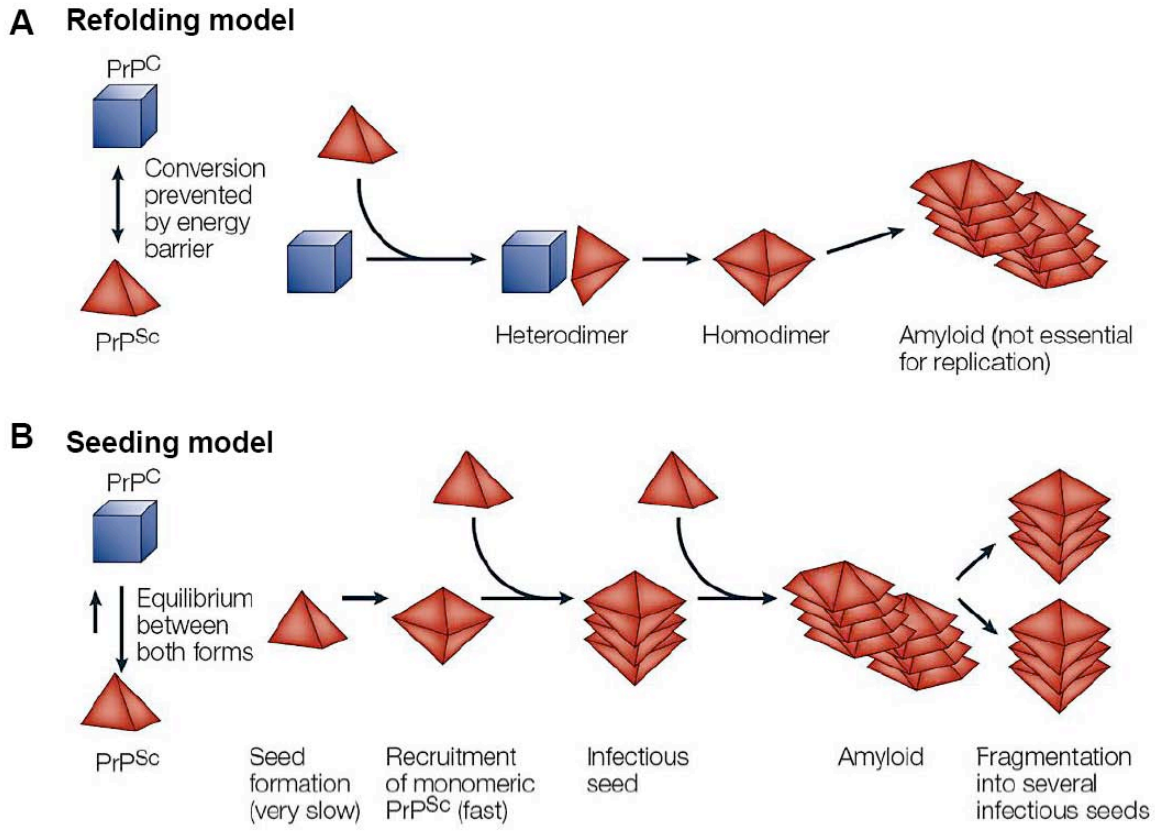


Figure 6

Models for the conformational conversion of PrP^C to PrP^{Sc}. A The "refolding" model postulates an interaction between exogenously introduced disease-associated prion protein (PrP^{Sc}) and endogenous cellular prion protein (PrP^C), which is induced to transform itself into more PrP^{Sc}. A high-energy barrier might prevent spontaneous conversion of PrP^C into PrP^{Sc}. **B** The "seeding" model proposes that PrP^C and PrP^{Sc} are in a reversible thermodynamic equilibrium. So, only if several monomeric PrP^{Sc} molecules are mounted in a highly ordered seed can more monomeric PrP^{Sc} be recruited and eventually aggregate to form an amyloid. In such crystal-like seed, PrP^{Sc} becomes stabilized. Fragmentation of PrP^{Sc} aggregates increases the number of nuclei, which can recruit more PrP^{Sc}, and so seems to result in replication of the agent. From Aguzzi *et.al.*, Nat Rev Immunol, 2004.

Site of conversion

The site of PrP^{Sc} generation is debated. PrP^{C}, the substrate for PrP^{Sc}, is a GPI-anchored protein located in lipid rafts that constitutively recycles between the cell surface and an endocytic compartment. Upon each recycling event, a fraction of PrP^{C} leaves the endocytic vesicles and is directed towards lysosomes for proteolysis. As part of its post-translational processing, PrP^{C} is cleaved within the GPI anchor, and the polypeptide chain is released into the extracellular matrix (Borchelt, Scott et al. 1990). Additionally, PrP^{C} has been detected in the nucleus and the cytoplasm.

The conversion of PrP^{C} into PrP^{Sc} could thus include any of these compartments. Evidence supports conversion taking place in lipid rafts, as PrP^{C} molecules bearing modifications in their cytoplasmatic tails leading to their redirection to clathrin pits were not converted (Taraboulos, Scott et al. 1995) (Kaneko, Vey et al. 1997). Other studies indicate that lipid rafts rather protect PrP^{C} against conversion to PrP^{Sc}. Raft depletion by inhibition of sphingomyelin production increased scrapie infection in cultured cells (Naslavsky, Shmeeda et al. 1999), and cholesterol depletion increased the proportion of misfolded PrP^{C} in the ER (Campana, Sarnataro et al. 2006) (Sarnataro, Campana et al. 2004). More experimental data is hence needed to clarify these contradictory findings.

Interestingly, PrP^{Sc} has been shown to accumulate in late exosomes, and increased exosome release increased the shedding of prions (Fevrier, Vilette et al. 2004) (Porto-Carreiro, Fevrier et al. 2005). However, the site of PrP^{Sc} accumulation may not reflect the compartment in which the conversion takes place.

3.2 Prion strains and species barrier

Like conventional micro-organisms, prion agents exhibit strain variation. A prion strain is defined by the reproducible incubation time, histopathological features and lesion distribution it induces in identical hosts. These characteristics are typically stable upon serial transmission in inbred mice (Aguzzi, Heikenwalder et al. 2007). Prion strains have also been shown to differ in their clinical manifestations (Wadsworth, Hill et al. 2003), their tropism (Aguzzi, Sigurdson et al. 2007), and their ease of transmission to other species (Caughey 2003). The molecular basis underlying prion strains is not known, but in line with the protein-only hypothesis, strain-specific information has been proposed to be encoded within the tertiary or quaternary structure of PrP^{Sc} molecules (Peretz, Scott et al. 2001) (Collinge and Clarke 2007). Supporting this idea is the finding that PK digestion of PrP^{Sc} from two different mink prion strains (drowsy and hyper) produces different PrP^{Sc} fragment sizes, suggesting that the two strains have different conformations resulting in the exposure of different PK cleavage sites (Bessen and Marsh 1994) (Bessen and Marsh 1992). Further structural analysis of prion strains using Fourier Transformed Infrared Spectroscopy (Aucouturier, Kascsak et al. 1999), conformation dependant immunoassays (Bellon, Seyfert-Brandt et al. 2003) and atomic force microscopy of synthetic prion polymers (Jones and Surewicz 2005) confirmed structural differences between prion strains. More recently, the use of a new series of conformation-specific protein dyes, called luminescent conjugated polymers (LCPs) (Nilsson, Herland et al. 2005), allowed to distinguish different prion strains based on the emission spectra of the dyes, further confirming the conformational differences between prion strains (Sigurdson, Nilsson et al. 2007).

Serial transmission of the infectious agent to inbred animals is required to formally define a prion strain. However, surrogate biochemical properties such as the fragment sizes of PrP^{Sc} after PK digestion (Collinge, Sidle et al. 1996) (Parchi, Castellani et al. 1996) and the prevalence of the three glycopatterns of PrP (Collinge, Sidle et al. 1996) are used when transmission experiments are not possible. Based on these biochemical properties, four types of sCJD, each associated with different clinicopathological phenotypes, have been defined (Collinge, Sidle et al. 1996) (Collinge, Sidle et al. 1996). How these conformational differences in PrP^{Sc} molecules relate to strain-specific disease phenotypes remains to be clarified.

Transmission of prion diseases between different species is typically far less efficient than within species. The incubation time is often longer and the attack rate reduced. This phenomena is called the species barrier (Collinge and Clarke 2007), and it was initially proposed to be due to PrP^C primary structure differences between donor and host. Supporting this suggestion is the finding that unlike wild-type mice, transgenic mice expressing hamster PrP^C are highly susceptible to hamster prions (Scott, Foster et al. 1989). However, transmission of sCJD prions to wild-type mice is very inefficient, while transmission of vCJD occurs much more readily (Hill, Desbruslais et al. 1997), although both strains share the same PrP^C primary structure. This suggests that to yet unknown features in addition to the difference in PrP^C primary structure affect the transmissibility of a prion strain to other species. It is suggested that PrP^C of each species is capable of a different range of PrP^{Sc} conformations, and that a given prion conformational variant can infect only those species for which the PrP^C can assume that same conformation (Collinge and Clarke 2007). However, in order to verify this hypothesis, the determinants governing the possible prion conformational variants that a particular PrP^C can assume must be elucidated.

3.3 A central nervous system disease with peripheral involvement

Lympho-reticular involvement in peripherally acquired prion diseases

Many TSEs result from peripheral exposure to prions that eventually spread to the brain where their toxicity is revealed. BSE, vCJD and kuru are all attributed to dietary exposure to prions. The routes of horizontal prion transmission of scrapie, CWD and TME are unknown, but transdermal and oral portals-of-entry have been postulated.

Studies in rodents have shown that after peripheral infection, an initial phase of prion replication takes place in secondary lymphoid organs, particularly the spleen and lymph nodes. Infectivity titres in the lymphoreticular tissues (LRT) reach a maximum well before infectivity is detectable in the brain, maintaining a plateau for the remainder of the disease progression (Aguzzi 2003) (Daude 2004). In the lymphoid tissue, Follicular dendritic cells (FDCs) are the main prion replication compartment (Klein, Frigg et al. 1997) (Prinz, Montrasio et al. 2002), but PrP^C-expressing haematopoietic cells are required for most efficient lymphoreticular prion propagation (Kaeser, Klein et al. 2001).

B cells provide FDCs with important cytokine stimuli such as membranous lymphotoxin (LT) $\alpha_1\beta_2$ and tumour-necrosis factor (TNF) α which maintain FDCs in their differentiated state (Mackay and Browning 1998). The absence of FDCs, that can be secondary to the absence of B cells or $LT\alpha_1\beta_2$ and $TNF\alpha$ signalling, delays neuroinvasion and reduces disease susceptibility (Mabbott, Young et al. 2003) (Mabbott, Mackay et al. 2000) (Montrasio, Frigg et al. 2000) (Bruce, Brown et al. 2000).

Depending on the host and the prion strain, the magnitude and duration of LRT involvement can vary considerably. In BSE, the prion titre in lymphoid tissue is typically low, and BSE is considered a neurotropic prion strain (Wells, Hawkins et al. 1998). vCJD, CWD, and many strains of scrapie, are lymphotropic (Aguzzi and Sigurdson 2004) and infectivity is detected at high levels both in the CNS and the LRT. Infectivity was detected in the tonsils of vCJD infected individuals (Wadsworth, Joiner et al. 2001), opening the way to a premortem diagnostic tool. However this finding also raises the concern of transmission of vCJD through surgical or diagnostic procedures.

Although prion accumulation in the secondary lymphoid organs may reach levels similar to those found in the CNS of end stage prion infected animals (Madec, Groschup et al. 2000), this process occurs without apparent damage to these organs.

Neuroinvasion

Neuroinvasion describes the process by which prions enter the CNS following peripheral entry in the organism. The exact mechanisms involved remain unclear, but the autonomic nervous system seems to play an important role in this process, as sympathectomy delays prion disease onset upon intra-peritoneal (i.p.) inoculation (Glatzel, Heppner et al. 2001). Moreover, reduction of the relative distance between FDCs and sympathetic nerves was shown to accelerate prion pathogenesis (Prinz, Heikenwalder et al. 2003). Once prions reach the spinal cord, they spread in both anterograde and retrograde directions (Beekes, Baldauf et al. 1996). Two hypothesis have been formulated to explain the mechanisms involved in prion axonal transport (Heikenwalder, Julius et al. 2007), but experimental data are missing to formally prove any of them.

Peripheral involvement in sporadic CJD

sCJD, the most frequent human prion disease is thought to result from PrP^{Sc} formation within the CNS. However, infectivity was also detectable in peripheral organs of sCJD patients, such as lymphoid tissue and muscle (Aguzzi and Sigurdson 2004) (Glatzel, Abela et al. 2003). These findings suggest that although a disease of the CNS, the presence of infectivity in peripheral organs must be taken into account when biosafety issues are discussed.

3.4 Mechanisms of neurodegeneration

The mechanisms leading to neuronal death in prion diseases are unknown. The absence of neurodegeneration in $Prnp^{0/0}$ mice argues strongly against neurotoxicity resulting from loss of function of PrP^{C} (Bueler, Fischer et al. 1992). Moreover, targeted PrP^{C} depletion in neurons of a fully developed mouse brain fails to induce a phenotype, ruling out acute loss of PrP^{C} as the cause of neurodegeneration in prion diseases (Mallucci, Ratte et al. 2002). However, the physiological function of PrP^{C} may be revealed in a pathological situation only.

It is tempting to postulate that PrP^{Sc}, the misfolded, PK resistant, and amyloidogenic isoform of PrP^{C} is toxic and that its accumulation is responsible for neuronal cell death. Supporting this hypothesis is the spatio-temporal correlation between the deposition of PrP^{Sc} in the brain and the appearance of neuropathological changes. PrP^{Sc} deposition typically appears prior to spongiosis and astrogliosis, which eventually develop in close vicinity to PrP^{Sc} (Jeffrey, Martin et al. 2001) (Williams, Lucassen et al. 1997) (Jendroska, Heinzel et al. 1991) (DeArmond, Mobley et al. 1987). However, a correlation is not always present and individual areas of the brain with strong PrP^{Sc} immunostaining but little or no neuropathological changes can be found (Budka 2003) (Hayward, Bell et al. 1994) (Parchi, Castellani et al. 1995).

Neurograft experiments have shown that PrP^{Sc} accumulation in the extracellular space is not toxic in the absence of PrP^{C}. *Prnp*$^{0/0}$ mice grafted with PrP^{C}-expressing embryonic neurons, and subsequently intracerebrally challenged with prions, accumulate substantial amounts of PrP^{Sc} in the graft and the adjacent *Prnp*$^{0/0}$ host tissue. While the graft undergoes typical spongiform degeneration, the adjacent *Prnp*$^{0/0}$ tissue in contact with PrP^{Sc} does not undergo neuropathological changes (Brandner, Isenmann et al. 1996). This suggests that PrP^{Sc} deposited extracellularily is not toxic to neurons in itself, but that neurodegeneration is instead the consequence of PrP^{Sc} formation within or at the surface of cells.

Neuronal depletion of PrP^{C} during the course of prion disease prevents clinical disease and reverses spongiosis, even though PrP^{Sc} levels in the brain remain high due to continuous production of PrP^{Sc} by astrocytes (Mallucci, Dickinson et al. 2003). Also, wild-type mice inoculated with hamster prion strain Sc237 propagate mouse-adapted prions but do not develop a clinical disease. The propagation occurs slowly, but prion

titres seen in end-stage conventional clinical disease are eventually reached. Second passage of these prions in mice or hamsters results in conventional transmission with short incubation periods and 100% lethality (Hill, Joiner et al. 2000).

More recently, transgenic mice expressing an anchorless version of PrP^{C} lacking the GPI signal and hence secreted in the extracellular space were shown to accumulate PrP^{Sc} plaques without developing clinical signs (Chesebro, Trifilo et al. 2005).

Taken together, these findings support a dissociation between PrP^{Sc} accumulation and toxicity, and indicate that molecules other than PrP^{Sc} such as intermediates in the prion replication process could be responsible for prion-induced neurodegeneration (Cohen, Pan et al. 1994).

4. Neurotoxicity associated with PrP^C deletion mutants and Doppel overexpression

4.1. *Prnp* knock-out mice and the discovery of Doppel

Doppel is a paralog of PrP^C discovered incidentally in 1999 following the generation of different *Prnp* knock-out (*Prnp*$^{0/0}$) mice and the development of a phenotype in some but not all lines. Several *Prnp*$^{0/0}$ mouse lines have been generated in an attempt to elucidate the physiological function of PrP^C. Two lines, called Zürich I (ZHI) (Bueler, Fischer et al. 1992) and Edinburgh (Edbg) (Manson, Clarke et al. 1994) are healthy except for minor electrophysiological (Collinge, Whittington et al. 1994) and circadian rhythm (Tobler, Gaus et al. 1996) anomalies. A peripheral demyelinating neuropathy is also observed in aged mice (Bueler, Fischer et al. 1992). Three other *Prnp*$^{0/0}$ lines develop cerebellar Purkinje cell degeneration (Sakaguchi, Katamine et al. 1996) (Rossi, Cozzio et al. 2001) (Moore, Lee et al. 1999) leading to ataxia in aged mice. The phenotype is rescued by co-expression of *Prnp*, suggesting initially that it is a consequence of the absence of PrP^C (Sakaguchi, Katamine et al. 1996) (Moore, Lee et al. 1999) (Rossi, Cozzio et al. 2001). The absence or presence of phenotype parallels the strategy used to generate the *Prnp*$^{0/0}$ lines. ZHI and Edbg were generated by replacing parts of the *Prnp* open reading frame (ORF) with a neomycin phosphotransferase gene (figure 7a). In the case of a third *Prnp*$^{0/0}$ line, called Nagasaki (Ngsk), 0.9kb of intron 2, the 5' non coding region and 0.45kb of the 3'non-coding region were deleted in addition to the *Prnp* ORF (Sakaguchi, Katamine et al.1996) (figure 7a). The phenotype observed in the Ngsk line was independently confirmed by two additional knock-out lines, Zürich II (ZHII) and Rcm0, in which the *Prnp* ORF and its flanking regions were replaced by a lox sequence and a hypoxanthine phosphoribosyltransferase (HPRT) cassette, respectively (Moore, Lee et al. 1999) (Rossi, Cozzio et al. 2001) (figure 7a). This suggested that the Nagasaki phenotype is not due to the absence of PrP^C, but rather to the cloning strategy. Sequence analysis of a cosmid containing *Prnp* revealed an ORF located 16kb downstream of *Prnp*, now known to code for Doppel (Dpl, for downstream of the *Prnp* locus, figure 7b). In the Ngsk, ZHII and Rcm0 lines, the deletion of intron 2 (including its splicing acceptor) leads to an intergenic splicing event, placing the Dpl locus under the control of the endogenous *Prnp* promoter (figure 7c). This intergenic splicing event is

also detected at very low levels in wild-type mice, but is greatly enhanced by the absence of the intron 2 splice acceptor (Moore, Lee et al. 1999) (Behrens and Aguzzi 2002) .

In contrast to PrP^C, Dpl is expressed at very low levels in the CNS of wild-type mice. In the Ngsk, ZHII and Rcm0 mice, Doppel gene (*Prnd*) mRNA is upregulated (Moore, Lee et al. 1999) (Lu, Wang et al. 2000) (Mo, Moore et al. 2001) (Silverman, Qin et al. 2000), leading to overproduction of Dpl in the brain of these $Prnp^{0/0}$ lines. An inverse correlation links *Prnd* mRNA levels to the onset of ataxia and increasing *Prnd* levels accelerate disease progression (Rossi, Cozzio et al. 2001). Deletion of *Prnd* from the ZHII mice abolishes the Nagasaki phenotype (Genoud, Behrens et al. 2004), confirming that the ectopic expression of Dpl in the CNS rather than the absence of PrP^C is responsible for the Purkinje cell loss.

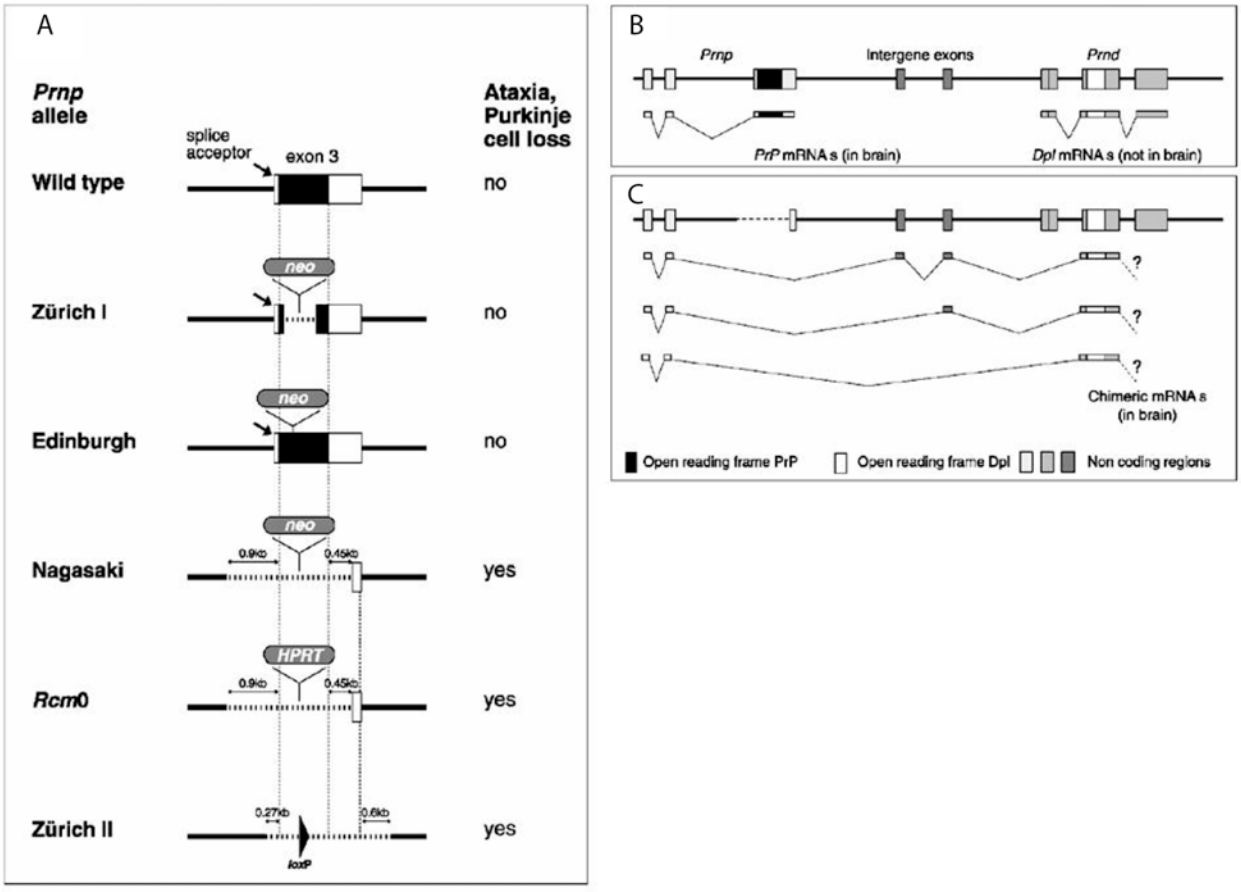


Figure 7

***Prnp* Knock-out strategies and their consequences**. **A** Various strategies used to target *Prnp* by homologous recombination. The black boxes represent PrP ORFs; white boxes, non-coding *Prnp* regions; grey boxes, inserted sequences; dotted line, deleted regions; neo, neomycin phosphotransferase; HPRT, hypoxanthine phosphoribosyltransferase; loxP (black arrowhead), a 34-bp recombination site from phage P1. **B** Coding and non-coding exons of *Prnp*, *Prnd* and intergenic exons of unknown function. **C** Exon skipping leads to expression of Doppel under the direction of the *Prnp* promoter. Deletion of the splice receptor site upstream of the third *Prnp* exon entails the formation of several chimeric mRNAs comprising the first two exons of *Prnp*, which are spliced directly or indirectly to the Dpl-encoding exon14. From Weissamnn *et al.*, Br Med Bull, 2003.

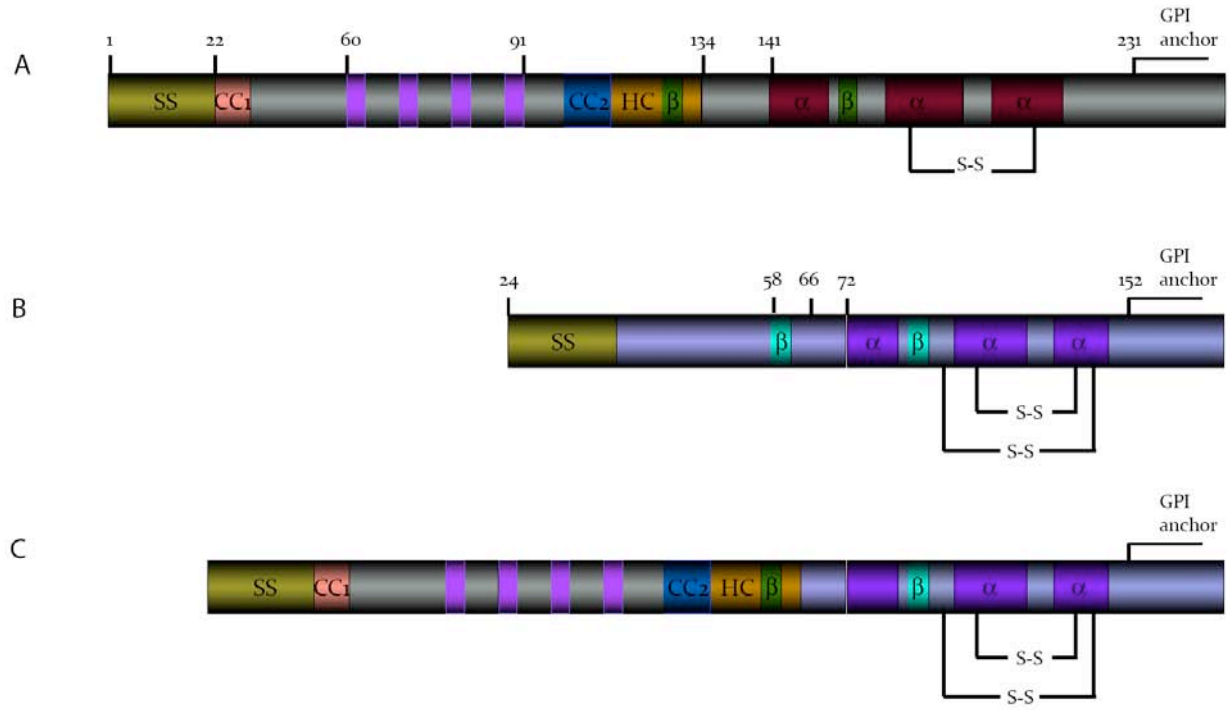


Figure 8

Structural features of the cellular prion protein, Doppel, and the chimeric protein Dpl_PrP. A Primary structure of the cellular prion protein.The numbers describe the position of the respective amino acids. SS (yellow) indicates the signal sequence; CC1 (pink) represents the charged cluster 1; OR (light blue) designates the octarepeat region; CC2 (dark blue) represents the charged cluster 2 and HC (orange) the hydrophobic core. The beta sheets are depicted in green and the alpha helices in red. S-S indicates the disulphide bridge. GPI denotes the membrane anchorage region. **B** Primary structure of Doppel. The numbers describe the position of the respective amino acids. SS (yellow) indicates the signal sequence. The beta sheets are depicted in green and the alpha helices in purple. S-S indicates the disulphide bridges. GPI denotes the membrane anchorage region. **C** Primary structure of a chimeric protein resulting from the fusion of Doppel with the first 134 amino acids of the prion protein. The numbers describe the position of the respective amino acids. SS (yellow) indicates the signal sequence; CC1 (pink) represents the charged cluster 1; OR (light blue) designates the octarepeat region; CC2 (dark blue) represents the charged cluster 2 and HC (orange) the hydrophobic core. The beta sheets are depicted in green and the alpha helices in purple. S-S indicates the disulphide bridges. GPI denotes the membrane anchorage region.

4.2 The neurotoxicity of Doppel and amino-truncated versions of PrP^C

The Dpl gene *Prnd* has been identified in many vertebrates including mouse (Moore, Lee et al. 1999), cattle, sheep (Tranulis, Espenes et al. 2001) and human (Moore, Lee et al. 1999) (Peoc'h, Guerin et al. 2000) and is highly conserved among mammals (Tranulis, Espenes et al. 2001). *Prnp* and *Prnd* bare no nucleic acid sequence homology and share only 24% identity in protein sequence (Mo, Moore et al. 2001) (Mastrangelo and Westaway 2001). However, the tertiary structure of Dpl is very similar to that of the carboxy-terminal two-thirds of PrP^C (Riek and Luhrs 2003). As PrP^C, Dpl is a GPI anchored protein harbouring 3 α-helices, two anti-parallel β-sheets and two facultative N-glycosylation sites (Mo, Moore et al. 2001). Dpl lacks a counterpart to the flexible N-terminal segment of PrP^C (Mastrangelo and Westaway 2001) (figure 8b).

The expression pattern of Dpl differs greatly from that of PrP^C. It is expressed at high levels in testis, at lower levels in other peripheral organs and at very low levels in the adult CNS (Moore, Lee et al. 1999). The generation of a *Prnd* knock-out mouse (*Prnd* $^{0/0}$) led to the identification of Dpl as a critical regulator of acrosome function in male gametogenesis (Behrens, Genoud et al. 2002).

There is no evidence that Dpl can become misfolded in presence of PrP^{Sc}. Indeed, in mice defective or overexpressing Dpl, neither the time course of prion disease nor the generation of PrP^{Sc} are significantly modified (Tuzi, Gall et al. 2002).

A neurotoxic phenotype similar to the one elicited by overexpression of Dpl in the CNS is observed in ZHI mice transgenically expressing amino-truncated versions of PrP^C. These mutants, called $PrP_{\Delta E}$ (Δ residue 32-121) and $PrP_{\Delta F}$ (Δ residue 32-134) and collectively referred to as $PrP_{\Delta N}$, harbour deletions including the octarepeat region and extending into the highly conserved hydrophobic core. While both Dpl overexpression in the CNS and transgenic expression of $PrP_{\Delta N}$ in ZHI mice lead to cerebellar neurodegeneration, cerebellar cortical neurons, and not Purkinje cells are the target of $PrP_{\Delta N}$ induced neurotoxicity. The absence of toxicity of $PrP_{\Delta N}$ in Purkinje cells can be explained by the vector driving the transgene expression (Fischer, Rulicke et al. 1996), which is not active in Purkinje cells (Shmerling, Hegyi et al. 1998) (Rossi, Cozzio et al. 2001). This is supported by the fact that targeted expression of $PrP_{\Delta F}$ to Purkinje cells leads to degeneration of these cells (Flechsig, Hegyi et al. 2003).

As in the case of Dpl overexpression in the CNS, the neurotoxicity induced by $PrP_{\Delta N}$ is abolished by co-expression of wild-type PrP^C (Flechsig and Weissmann 2004) (Flechsig, Shmerling et al. 2000).

Transgenic expression of two PrP mutants with an intact octarepeat region but harbouring truncations in the central domain leads to a highly neurotoxic phenotype. The neurotoxicity arising from $PrP_{\Delta 105\text{-}125}$ (Li, Christensen et al. 2007) expression is similar to the one elicited by $PrP_{\Delta E}$ and $PrP_{\Delta F}$, yet much stronger. Indeed, supraphysiological levels of wild-type PrP^C are needed to reduce the cortical neuron death and attenuate the leukoencephalopathy, but fail to rescue the phenotype completely (Li, Christensen et al. 2007). The expression of $PrP_{\Delta CD}$ (Δ 94-133) (Baumann, Tolnay et al. 2007) which lacks the whole central domain, elicits a predominantly white matter disease, with central and peripheral axonomyelinic degeneration. Supraphysiological levels of PrP^C are required to abolish the phenotype.

All proteins eliciting a neurotoxic phenotype bear deletions extending into the hydrophobic core, identifying the disruption of this highly conserved domain as determinant in the development of a neurotoxic phenotype (Shmerling, Hegyi et al. 1998). The phenotype they elicit is rescued by coexpression of various levels of full-length PrP^C, indicating that a same molecular mechanism underlies their neurotoxicity.

It is suggested that PrP^C interacts with a yet unknown receptor (PrP_R) to deliver an essential signal. Neurotoxicity arises from the binding of PrP-like molecules Dpl or $PrP_{\Delta N}$ to PrP_R without delivery of the signal (Weissmann and Aguzzi 1999). Confusingly, the expression of a third mutant, $PrP\Delta_{pHC}$, which lacks eight residues in the hydrophobic core (Δ 114-121) is not intrinsically toxic but exacerbates the phenotype of $PrP_{\Delta CD}$ while attenuating that of $PrP_{\Delta F}$. A model taking into account all the mutants discussed here has been suggested by Baumann *et al* (Baumann, Tolnay et al. 2007). However, the identification of the hypothetical PrP receptor PrP-R is required to verify this model.

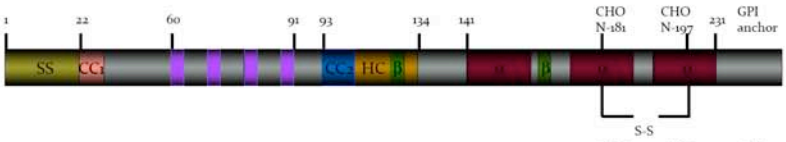
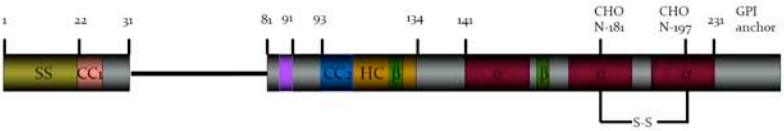
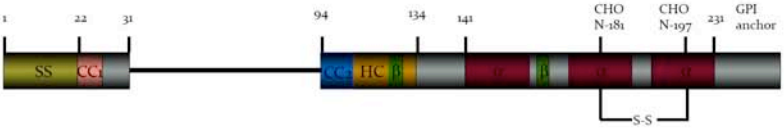
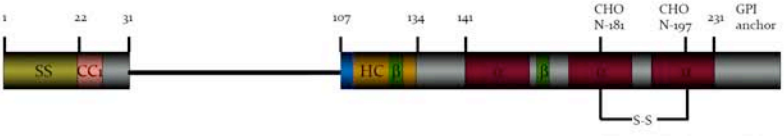
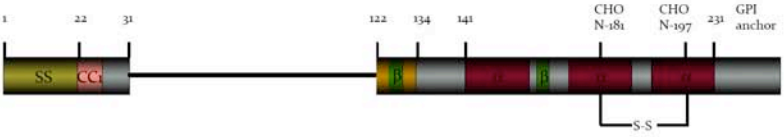
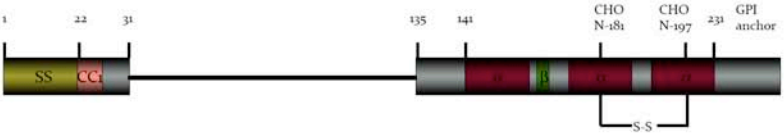
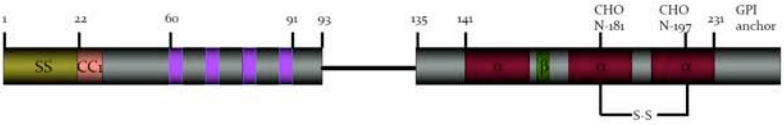
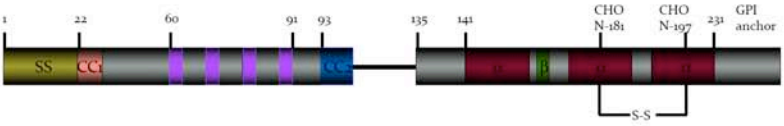
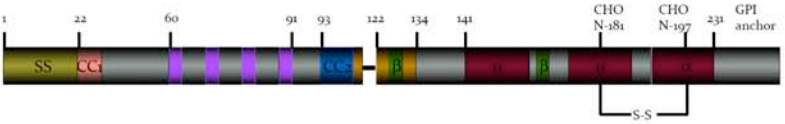
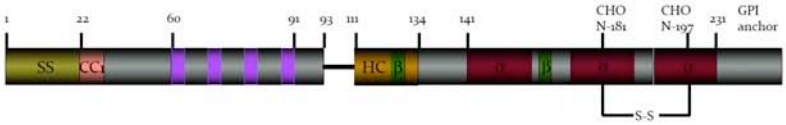
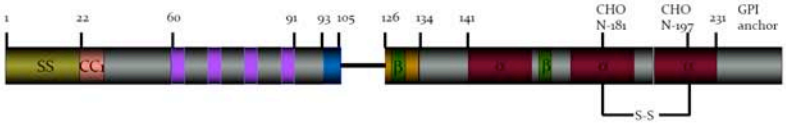
		Phenotype	Prion propagation	Ref.
wild-type				
PrPΔB del. 32-80		no	yes	a
PrPΔC del. 32-93		no	yes	b,c
PrPΔD del. 32-106		no	no	b,d
PrPΔE del. 32-121		cerebellar disorder	no	b
PrPΔF del. 32-134		cerebellar disorder	no	b,d
PrPΔCD del. 94-134		cerebellar disorder	nd	f
PrPΔHC del. 111-134		cerebellar disorder	nd	g
PrPΔpHC del.114-121		no	no	f,i
PrPΔCC2 del.94-110 .		no	nd	g
PrPΔ105–125 del.105-125		cerebellar disorder	nd	h

Figure 9

Structural features of the cellular prion protein and the amino-terminal deletion mutants. Primary structure of the cellular prion protein including post-translational modifications. The numbers describe the position of the respective amino acids. SS (yellow) indicates the signal sequence; CC1 (pink) represents the charged cluster 1; OR (light blue) designates the octarepeat region; CC2 (dark blue) represents the charged cluster 2 and HC (orange) the hydrophobic core. The beta sheets are depicted in green and the alpha helices in red. S-S indicates the disulphide bridge. GPI denotes the membrane anchorage region.
Ref. a: (Fischer, Rulicke et al. 1996). Ref. b : (Shmerling, Hegyi et al. 1998). Ref. c: (Flechsig, Shmerling et al. 2000). Ref. d: (E. Flechsig). Ref. f: (Baumann, Tolnay et al. 2007). Ref.g: (Baumann). Ref. h: (Li, Christensen et al. 2007)

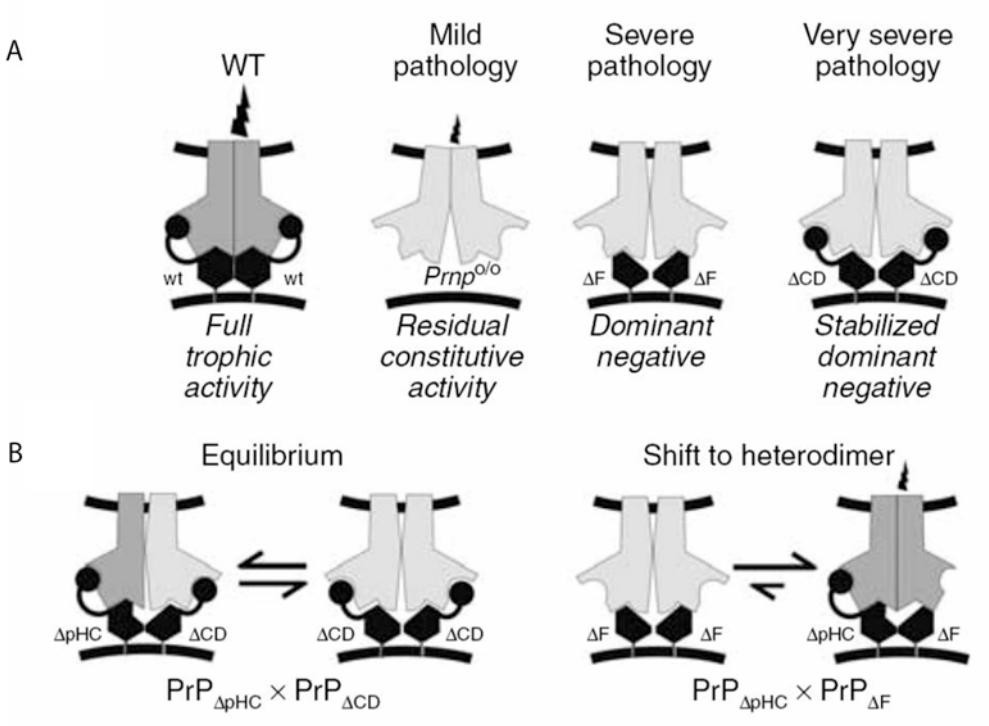


Figure 10

A model for the positive and negative effects of PrP^C and its variants. A PrP (black) consists of a globular C-terminal domain (hexagon) and an N-terminal flexible tail (arch) encompassing the ORs (circle). The model rests on the following assumptions: (1) PrP^C activates a hitherto unidentified receptor (PrP_R), which transmits myelin maintenance signals (flashes); (2) in the absence of PrP^C, PrP_R exerts some residual activity, either constitutively or by recruiting a surrogate ligand; (3) the activity of PrP^C and its mutants requires homo- or heterodimerization, and induces dimerization of PrP_R; and (4) PrP dimers containing $PrP_{\Delta CD}$ or $PrP_{\Delta F}$ trap PrP_R in an inactive dominant-negative state. Finally, (5) the OR region stabilizes the interaction between PrP and PrP_R, but does not contribute directly to signalling. **B** By increasing complex affinity to PrP_R through its ORs, $PrP_{\Delta pHC}$ may displace $PrP_{\Delta F}$ homodimers, thereby ameliorating the phenotype of compound $PrP_{\Delta F\Delta pHC}$ transgenic mice. However, as $PrP_{\Delta CD}$ also contains ORs, it may more efficiently compete with $PrP_{\Delta pHC}$ for PrP_R and thus prevents any phenotypic improvement.

II. Generation of a transgenic mouse to study the role of the flexible amino-terminal domain in the physiological function of PrP^C

1. Summary and outline of the study

Mice devoid of PrP^C (*Prnp*$^{0/0}$) develop normally and fail to exhibit a phenotype in the CNS. However, transgenic mice expressing amino-truncated versions of PrP^C with deletions extending into the hydrophobic core (collectively termed $PrP_{\Delta N}$) suffer from neurodegeneration, suggesting a role for this domain in a survival pathway. One of such mutants, $PrP_{\Delta CD}$, is particularly toxic, leading to death of *Prnp*$^{0/0}$ mice around postnatal day 25.

Doppel, a protein structurally similar to the carboxy-terminal domain of PrP^C (figure 8b) and physiologically expressed in the testis, is toxic when expressed in the CNS, eliciting a phenotype similar to that induced by $PrP_{\Delta N}$ expression.

In both cases, neurodegeneration is rescued by co-expression of full-length PrP^C, suggesting all proteins are involved in the same pathway and compete for interaction with a yet unknown receptor (PrP_R).

A chimeric protein consisting of Doppel fused to the amino-terminal domain of PrP^C, termed Dpl_PrP (figure 8c), is not toxic and can rescue the neurodegeneration induced by $PrP_{\Delta N}$ (Baumann et al, manuscript in preparation). A similar construct was recently shown to rescue the neurotoxicity of Doppel expression in the CNS (Yoshikawa, Yamaguchi et al. 2008). These findings confirm the essential role of the amino-terminus of PrP^C in conveying the activity of the full-length protein.

To understand the contribution of the globular domain to the physiological activity of the full-length protein, we generated a transgenic mouse expressing a redacted version of the prion protein, lacking the entire carboxy-domain ($PrP_{\Delta 141-231}$).

We crossed this mouse with two transgenic lines expressing $PrP_{\Delta CD}$, and show that co-expression of $PrP_{\Delta 141-231}$ does not modify the toxic phenotype induced by $PrP_{\Delta CD}$, although both proteins are expressed in the same compartment (lipid rafts) and at similar levels.

However, the results of the rescue experiments must be interpreted with caution. Indeed, while a low amount of a toxic mutant ($PrP_{\Delta CD}$) with a potentially high affinity for PrP_R induces a phenotype, the co-expression of an equimolar amount of a protein devoid of the globular domain might not suffice to compete for interaction with PrP_R. The absence of rescue may thus rather be due to the reduced affinity of $PrP_{\Delta 141\text{-}231}$ for PrP_R and an increased molar ratio of $PrP_{\Delta 141\text{-}231}$ to $PrP_{\Delta CD}$ may be required to compete for interaction with PrP_R.

2. Results

2.1 Expression of the transgene in two different murine neuronal cell lines gives rise to a stable protein located in lipid rafts

2.1.1 Generation of the transgene

To investigate the contribution of the carboxy-domain of PrP^C to the physiological function of the full-length protein, we generated a transgene coding for a truncated murine PrP^C variant lacking the carboxy-terminal globular domain. To direct the protein to the same subcellular compartment as the full-length protein, we fused the amino-terminal domain to the GPI anchor signal of PrP^C. The resulting protein, called $PrP_{\Delta 141-231}$, consists of the first 140 amino acids of PrP^C directly linked to the last 23 amino acids of the full length polypeptide which are replaced, in the mature protein, by a GPI anchor (figure 11).

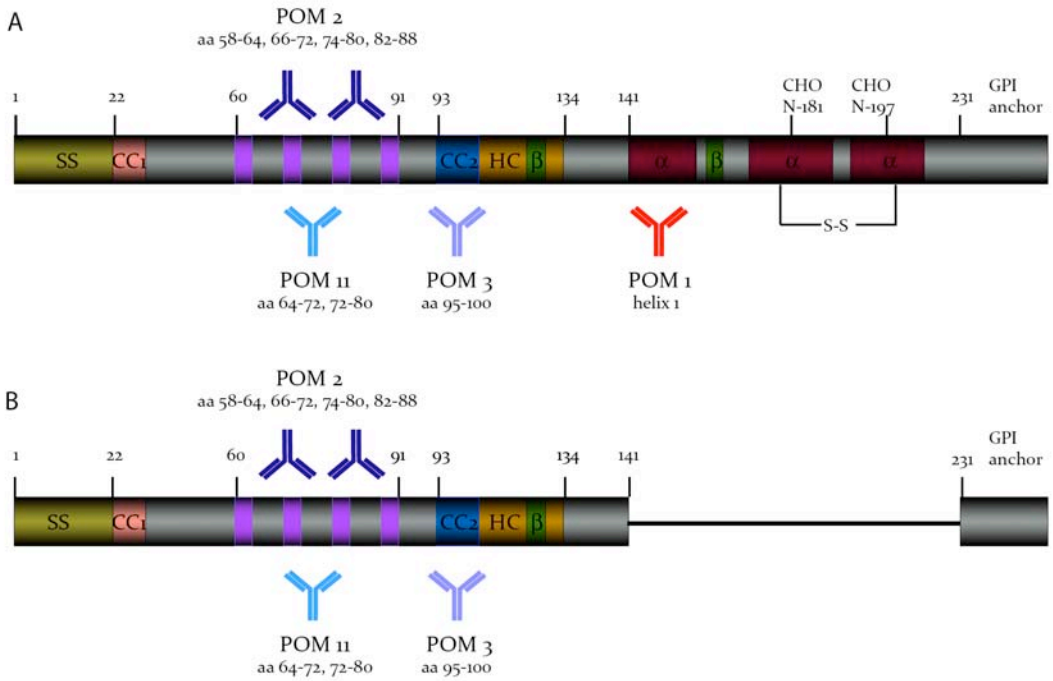


Figure 11

Primary structure of murine PrP^C. The numbers describe the positions of the amino-acids. SS (yellow) designates the signal sequence; CC1 (pink) represents the charged cluster 1; OR (purple) the four octarepeats; CC2 (dark blue) the charged cluster 2; HC (orange) the hydrophobic core; β (green) the two β-sheets and α (red) the three α-helices. GPI anchor (glycosyl-phosphatidyl-inositol anchor).The N-terminus of PrP^C spans from amino-acid 1 to 140. It includes a short β-sheet (amino-acids 128-131) within the hydrophobic core. The structured C-terminal domain starts at the first α-helix and ends at amino-acid 231, were the GPI anchor is added. Two facultative N-glycosylation sites exist (amino-acids 181 and 197), giving rise to three PrP^C species: un-, mono- and di-glycosylated PrP^C. The anti-PrP antibodies POM2, POM3 and POM11 are illustrated in blue. The anti-PrP antibody POM1 (illustrated in red) recognizes an epitope within the first α-helix. **A** Full length murine PrP consists of 231 amino-acids. **B** PrP$_{\Delta 141\text{-}231}$: the first 140 amino-acids of PrP are directly linked to the GPI recognition site.

2.1.2 Cloning strategy

The strategy used for the construction of the transgene is described in the materials and methods section. Briefly, total mouse brain cDNA was used as a template, and appropriately designed primers were employed to amplify and fuse the amino-terminus of full length PrP^C with the GPI signal sequence of the protein.

2.1.3 Expression of the transgene in two neuronal cell lines

Wild-type PrP^C and $PrP_{\Delta 141\text{-}231}$ sequences were inserted into an expression plasmid (pBMN). The resulting plasmids were transfected into Hpl and N2a cells. The Hpl cell line is derived from hippocampal neurons isolated from Ngsk mice, and hence does not express *Prnp*, while the neuroblastoma-derived cell line N2a does.

Full length unglycosylated PrP^C is a 25 kD protein consisting of 209 amino-acids (figure 12, lane 6). $PrP_{\Delta 141\text{-}231}$ consists of 118 amino acids and lacks the 2 facultative N-glycosylation sites that are located in the globular carboxy-terminus of full length PrP^C. We found that $PrP_{\Delta 141\text{-}231}$ is expressed as an unglycosylated 15kD protein in Hpl (figure 12, lane 1 and 4). To assess the integrity of $PrP_{\Delta 141\text{-}231}$, we verified the presence of epitopes of three amino-terminal anti-PrP antibodies (POM11, POM3 and POM2, figure 11). All epitopes were present in $PrP_{\Delta 141–231}$ (data not shown). However, these findings cannot exclude a truncation of the protein amino-terminally to the OR region (POM12 epitope; figure 11) or carboxy-terminally to the central domain (POM3 epitope; figure 11).

The presence of the GPI anchor was assessed by comparing the electrophoresis mobility of $PrP_{\Delta 141–231}$ with that of an anchorless version of the same protein (called $sPrP_{\Delta 141–231}$ for secreted $PrP_{\Delta 141–231}$) on a SDS-PAGE. The slight difference in the electrophoresis mobility of the two proteins suggested the presence of a GPI anchor in $PrP_{\Delta 141–231}$ (figure 13).

In N2a cells, expression of the transgene gave rise to the same 15kD band and an additional, higher molecular weight band (figure 13, lane 2). The identity of this second band is unknown, but the two species do not correspond to a GPI anchor plus and minus version of the same protein, since $sPrP_{\Delta 141–231}$ runs at a lower molecular weight on a SDS-PAGE (figure 13, lane 4).

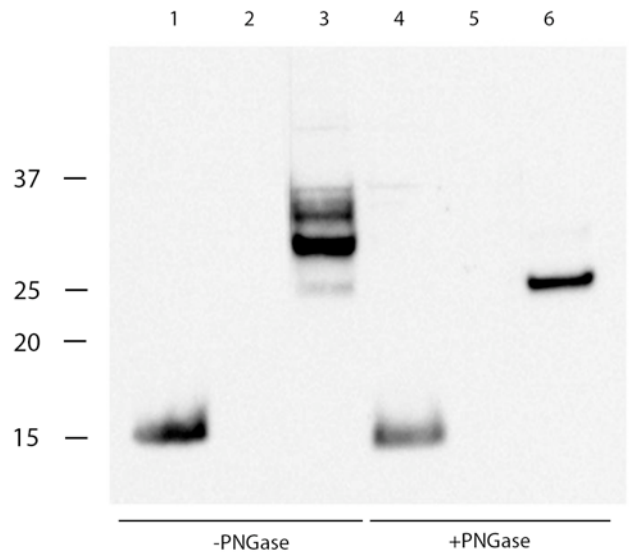


Figure 12

Expression of $PrP_{\Delta 141-231}$ in Hpl cells gives rise to a 15kD protein. Lane 1 and 4, cell lysate of Hpl cells transfected with $PrP_{\Delta 141-231}$. Lane 2 and 5 cell lysate of Hpl cells trasfected with the empty vector. Lane 3 and 6, cell lysate of Hpl cells transfected with wild-type PrP. Lane 4 to 6: cell lysate subjected to peptide-N-glycanase (PNGase) digestion. Western blot probed with POM11 (see figure 11).

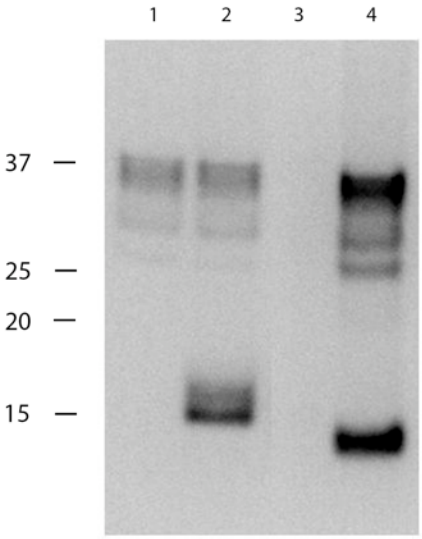


Figure 13

Expression of $PrP_{\Delta 141-231}$ in N2a cells. Lane 1: cell lysate of N2a cells transfected with the empty vector. The endogenous PrP^{C} is seen between 25 and 37kD. Lane 2: cell lysate of N2a cells transfected with the $PrP_{\Delta 141-231}$ construct. Lane 3: cell lysate of untransfected Hpl cells. Lane 4: cell lysate of N2a cells transfected with $sPrP_{\Delta 141-231.}$ Western blot probed with POM11 (see figure 11).

2.1.4 $PrP_{141-231}$ is located in lipid rafts in Hpl cells

The buoyancy of $PrP_{\Delta 141-231}$ in a step density gradient centrifugation was similar to that of PrP^C and flotillin, indicating that $PrP_{\Delta 141-231}$ resides in lipid rafts of Hpl cells (figure 14). These results show that a PrP^C variant lacking most of its structured domain can be successfully expressed *in vitro*, and that this protein is located in similar membrane microdomains as the full length protein.

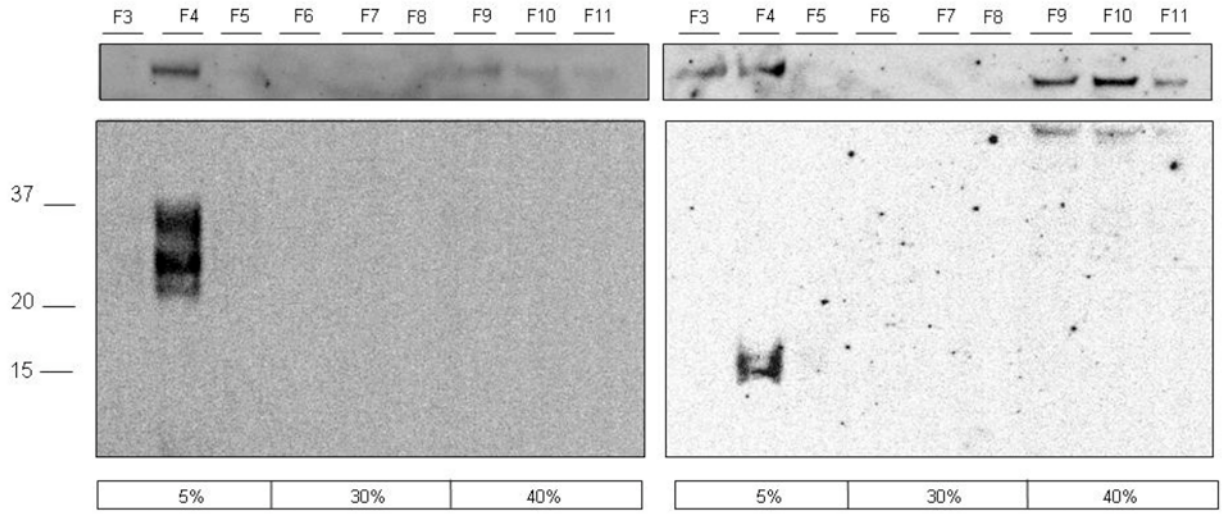


Figure 15

$PrP_{\Delta 141-231}$ colocalizes with flotillin in lipid rafts. Left panel: Hpl cells transfected with the $PrP_{\Delta 141-231}$ sequence. Right panel: Hpl transfected with PrP wild-type sequence. The lower blots were probed with POM2 (see figure 11) and the upper blots with an anti-flotillin antibody.
Fractions 3 to 5 (F3-F5): 5% Optiprep. Fractions 6 to 8 (F6-8): 30% Optiprep. Fractions 9 to 11 (F9-11): 40% Optiprep.

2.2 Generation of a transgenic mouse expressing $PrP_{\Delta 141\text{-}231}$

2.2.1 The expression vector

Based on the results obtained in the cell culture experiments, we proceeded to the generation of a transgenic mouse expressing $PrP_{\Delta 141–231}$.

The vector used to drive the expression of the transgene *in vivo* is the “half genomic vector” (Fisher 1996). It encompasses the entire murine *Prnp* locus, with 6 kb and 2.2 kb of 5’ and 3’ untranslated sequences, respectively. The second intron of *Prnp* is deleted, fusing exons 2 and 3 (figure 16). The vector directs orthoptic expression of *Prnp* to almost all cell types in which PrP^C is expressed. One notable exception are cerebellar Purkinje cells.

The open reading frame (ORF) of PrP^C was replaced by that of $PrP_{\Delta 141–231}$ in the half-genomic construct. The cloning strategy is described in the materials and methods section.

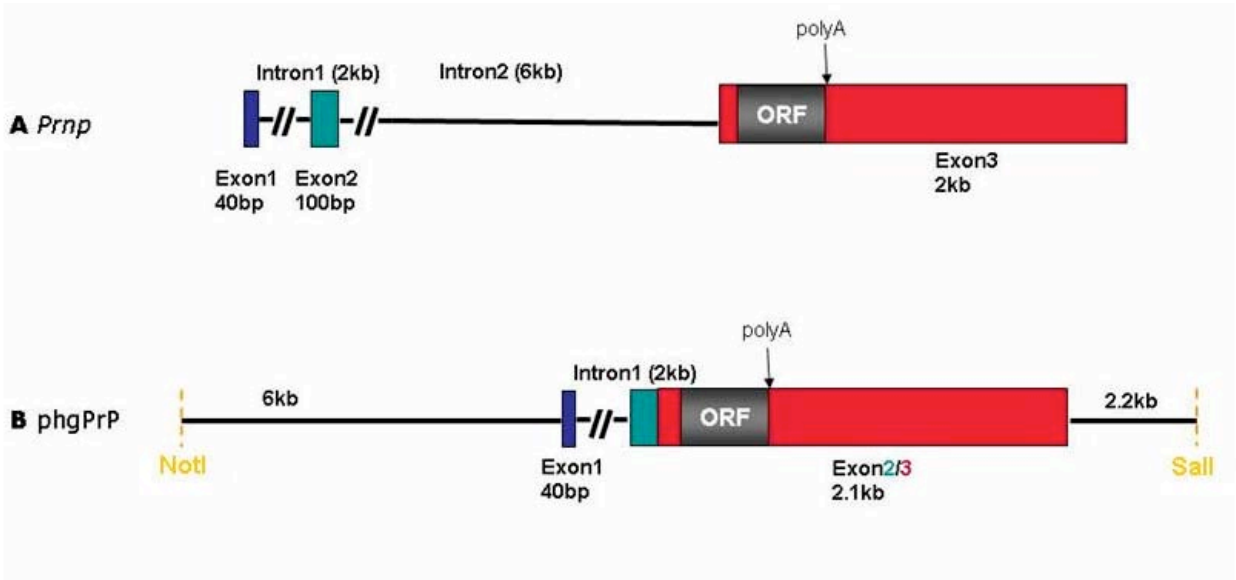


Figure 16

Murine *Prnp* and the half-genomic construct. Exons are represented as boxes. Exon 1 (blue box) is separated from exon 2 (green box) by intron 1. **A.** In *Prnp*, intron 2 separates exon 2 from exon 3 (red). **B.** In the half-genomic construct (phgPrP), intron 2 is deleted and exon 2 and 3 are fused.

2.2.2 Pronuclei microinjections

The half-genomic construct containing the $PrP_{\Delta 141-231}$ ORF was injected into pronuclei of *Prnp*$^{+/+}$ mice in the laboratory of Thomas Rülicke in Vienna.

Sixty potential founders were screened by tail PCR for the presence of the transgene. Fourteen mice were transgene positive. Three transgenic lines (*tg*155, *tg*157 and *tg*177) were selected on the basis of the protein expression level of the transgene in the CNS (figure 17). The highest expresser line, *tg*157, was unfortunately lost. The founder and its positive offspring died before further analysis could be performed.

The two remaining lines (*tg*155 and *tg*177) were crossed with *Prnp*$^{0/0}$ mice to obtain transgenic positive offspring on a *Prnp*$^{0/0}$ background. Both male and female offspring were born with Mendelian frequencies and had a normal life span.

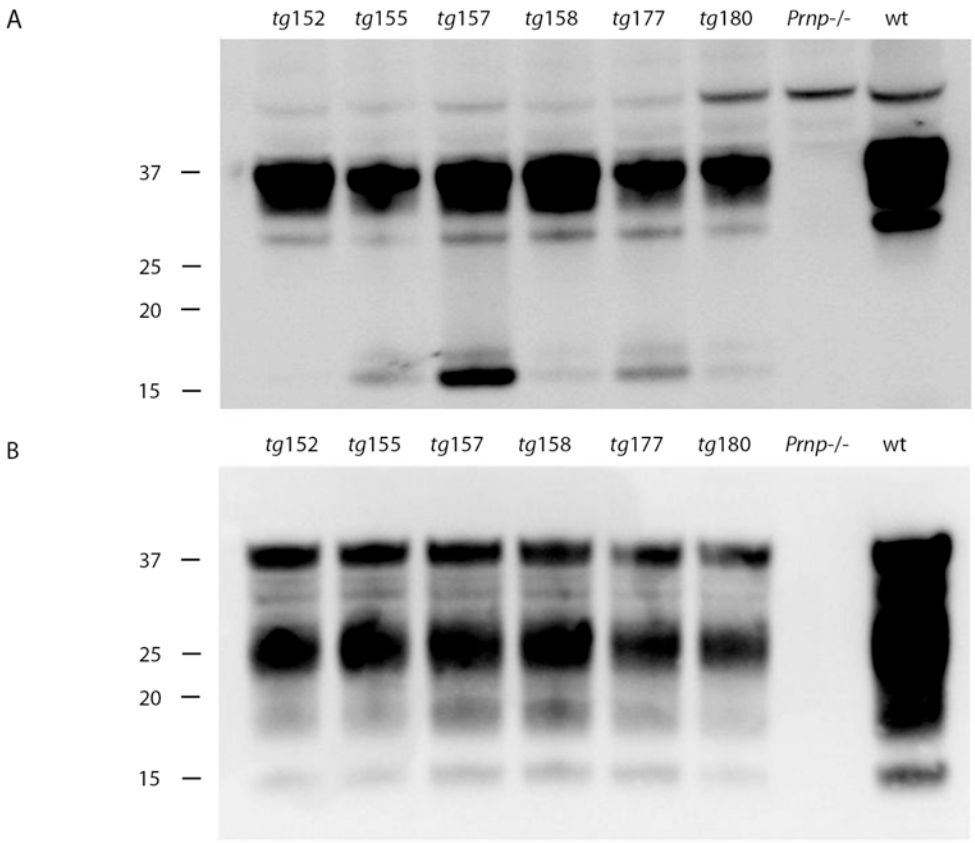


Figure 17

Transgene expression analysis in the brain of six transgenic lines. Western blot of brain homogenate of six transgenic lines (*tg*152, *tg*155, *tg*157, *tg*158, *tg*177 and *tg*180) on a *Prnp*$^{+/-}$ background, and *Prnp*$^{0/0}$ and wild-type (wt) controls. **A** Blot probed with POM11, an amino-terminal antibody (see figure 11). The wild-type allele gives rise to three bands between 37 and 25 kD (di-, mon-, and unglycosylated PrP^{C}). The transgene gives rise to two bands around 15kD **B** Duplicate of A, probed with POM1, a carboxy-terminal antibody (see figure 11). POM1 does not recognise the transgene, but detects the wild-type allele and the carboxy-terminal cleavage products of PrP^{C}, amongst which unglycosylated C1 (see figure 5), which runs at about 15 kD.

2.2.3 The transgene copy number in line *tg*155 and *tg*158

Southern blot analysis was performed for line *tg*155 and *tg*158 and revealed a transgene copy number of 12 and 18, respectively (figure 18 C). The protein expression of *tg*158 being low, the line was eliminated.

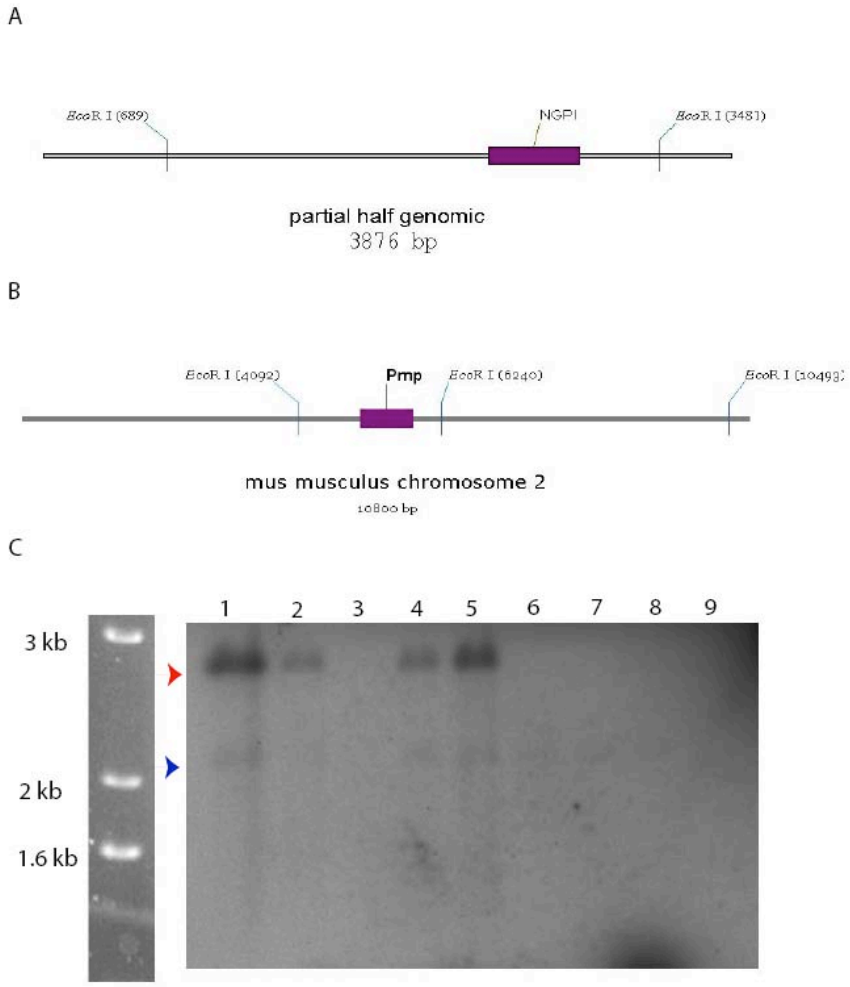


Figure 18

Determination of transgene copy number in two transgenic lines. A Transgene (half-genomic) displaying EcoRI restriction sites. Digestion with EcoRI gives rise to a 2792bp fragment. **B** Murine *Prnp* displaying EcoRI restriction sites. Digestion with EcoRI gives rise to a 2148bp fragment. **C** Southern blot of two transgenic lines *tg*155 and *tg*177. Lane 1 and 2: *tg*155 on a $Prnp^{+/-}$ background. Lane 3: empty. Lane 4 and 5: *tg*158 on a $Prnp^{+/-}$ background. Lane 6 and 7: wild-type. Lane 8 and 9: $Prnp^{0/0}$, which does not anneal with the probe. The wild-type allele (2148bp) is indicated with a blue arrowhead and the transgene (2792bp) with a red arrowhead.

2.2.4 $PrP_{\Delta 141\text{-}231}$ expression in the brain of *tg*155 and *tg*177 is low

Expression of the transgene *in vivo* gave rise to two bands migrating at 15 and 16kD in an SDS-PAGE (figure 19), similarly to what was seen in N2a cells transfected with the $PrP_{\Delta 141\text{-}231}$ construct (figure 13). Both bands were detectable with three amino-terminal anti-PrP antibodies POM2, POM3 and POM11 (data not shown). The two bands were

also detectable in primary cortical neurons isolated from E 15.5 *tg*155 mice (data not shown).

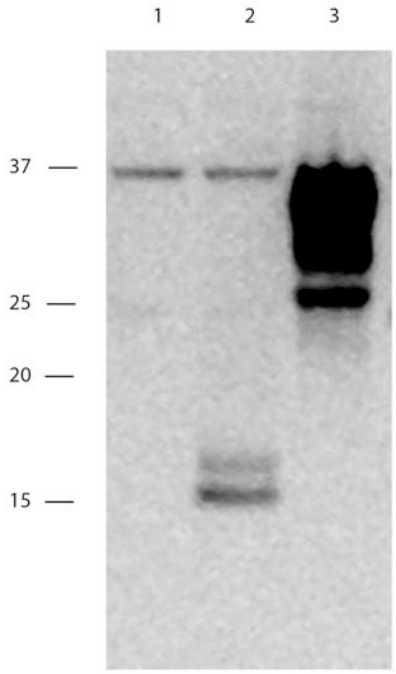


Figure 19

Transgene expression analysis in the brain of *tg*155. Western blot of brain homogenate of *Prnp*$^{0/0}$ (lane 1), *Prnp*$^{0/0}$ *tg*155 (lane 2) and wild-type (lane 3) mice. Blot probed with POM2, an amino-terminal antibody (see figure 11). The wild-type allele gives rise to three bands between 37 and 25 kD (di-, mon-, and unglycosylated PrP^{C}). The transgene gives rise to two bands around 15kD. An unspecific band is seen at 37kD in all three samples including the *Prnp*$^{0/0}$ control.

2.2.5 $PrP_{\Delta 141-231}$ is located in lipid rafts in the CNS

In order to be able to observe a potential rescue of the neurotoxic phenotype seen in transgenic mice expressing $PrP_{\Delta N}$ variants, $PrP_{\Delta 141-231}$ has to be localized in the same subcellular compartment as $PrP_{\Delta N}$ and PrP^{C}.

We found that both bands resulting from the expression of the $PrP_{\Delta 141-231}$ construct were localized in lipid rafts in the mouse brain. However, while PrP^{C} is mostly recovered in fraction 4, $PrP_{\Delta 141-231}$ is detected in fraction 5, suggesting the two proteins could be located in different types of lipid rafts (figure 20).

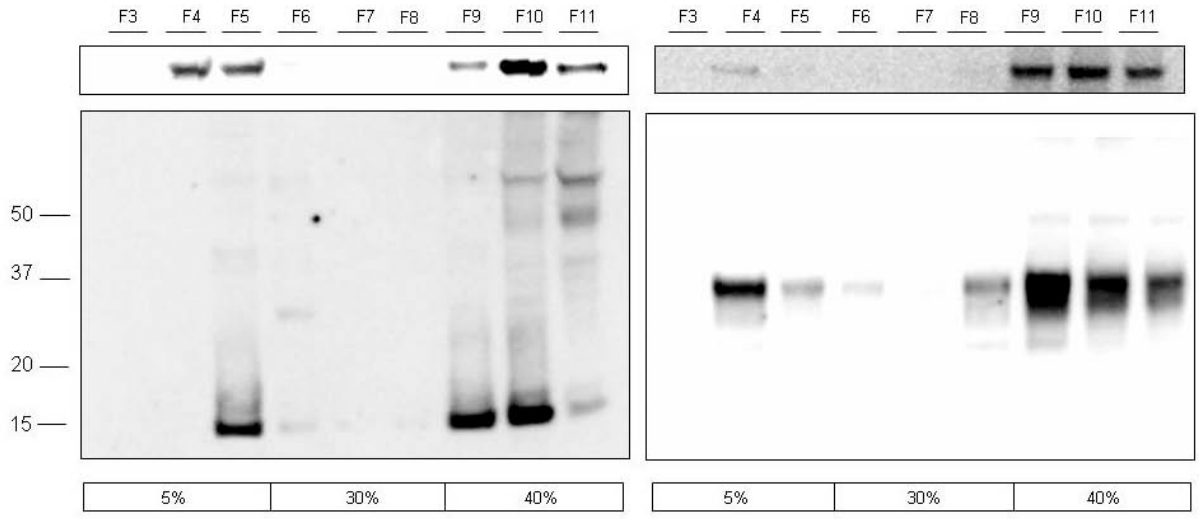


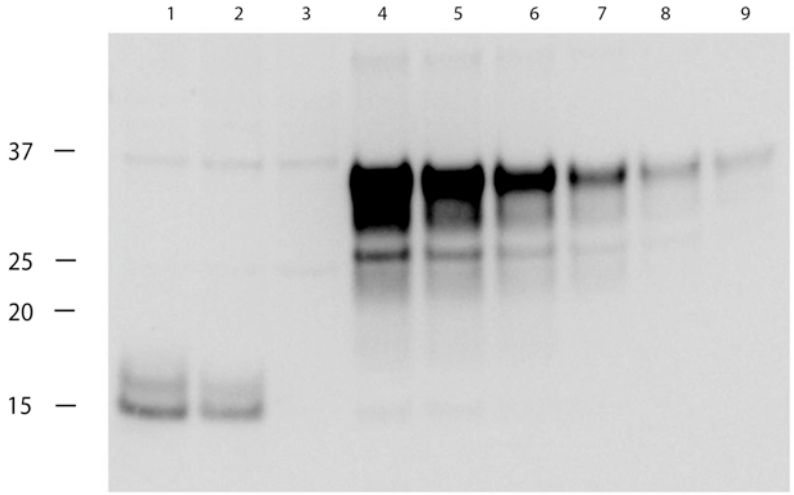
Figure 20

$PrP_{\Delta 141-231}$ colocalizes with flotillin in lipid rafts. Left panel: Brain homogenate of *tg*155 on a $Prnp^{-/-}$ background. Right panel: Brain homogenate of a wild-type mouse. The lower blots were probed with POM11 (see figure 11) and the upper blots with an anti-flotillin antibody.
Fractions 3 to 5 (F3-F5): 5% Optiprep. Fractions 6 to 8 (F6-8): 30% Optiprep. Fractions 9 to 11 (F9-11): 40% Optiprep.

2.2.6 Quantification of the transgene expression

We compared the expression levels of $PrP_{\Delta 141\text{-}231}$ to that of endogenous PrP^{C} in the brain of wild-type mice by quantitative Western blotting. Serial dilutions of wild-type brain homogenate were blotted (figure 21A), and the relative expression levels of the two molecules were calculated from calibration curves obtained by direct acquisition of chemiluminescence (figure 21B). In total brain homogenate, we found that *tg*155 and *tg*177 expressed $PrP_{\Delta 141\text{-}231}$ at a level corresponding to 7% and 9% respectively of a *Prnp*$^{+/+}$ mouse (Figure 21).

Quantification of RNA expression revealed that the relative transcript copies of *tg*155 to wild-type was 18 times higher (figure 22), suggesting that $PrP_{\Delta 141\text{-}231}$ is less stable than the full length protein.



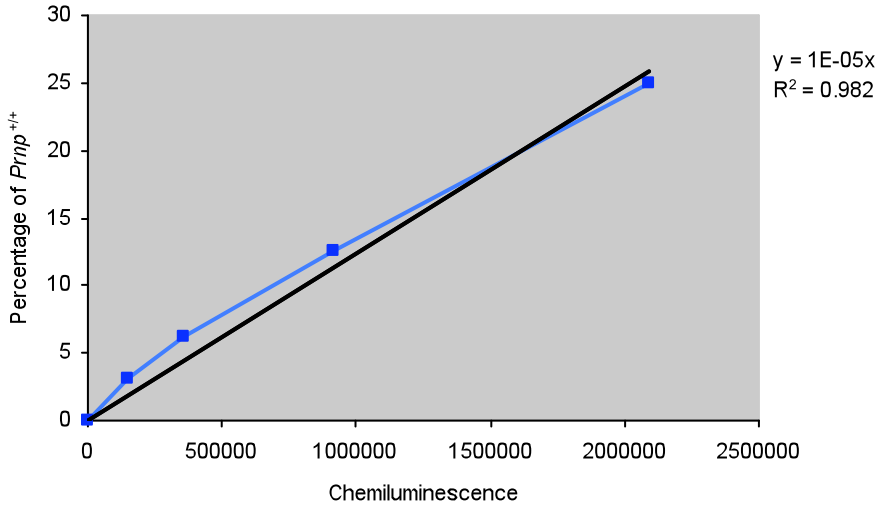


Figure 21

Quantification of $PrP_{\Delta 141-231}$ in the brain of *tg*155 and *tg*177 compared to PrP^C in wild-type mouse brain. Upper panel: western blot probed with POM11 (figure 11). Lane 1: 20ug protein of a *tg*155 (*Prnp*$^{-/-}$ background) brain homogenate. Lane 2: 20ug protein of a *tg*177 (*Prnp*$^{-/-}$ background) brain homogenate. Lane 3: 20ug protein of a *Prnp*$^{-/-}$ brain homogenate. Lane 4: 20ug protein of a *Prnp*$^{+/+}$ brain homogenate. Lane 5-9: serial 1:2 dilution starting with the amount of protein in lane 4. Lower panel

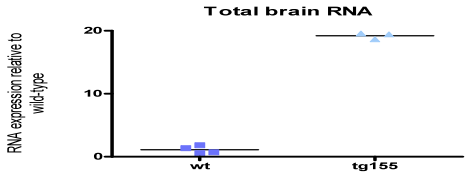


Figure 22

Brain RNA levels of $PrP_{\Delta 141-231}$ in *tg*155 compared to the RNA levels of PrP^C in a wild-type mouse brain. The RNA levels of $PrP_{\Delta 141-231}$ are 18 times higher than that of PrP^C in a wild-type mouse brain, paralleling the transgene copy number in *tg*155.

2.2.7 $PrP_{\Delta 141-231}$ has a reduced half-life *in vivo*

To verify if the discrepancy between the high RNA levels and the relatively low protein levels in the brain of *tg*155 and *tg*177 mice were due to a decreased stability of $PrP_{\Delta 141-231}$, we measured the half-life of $PrP_{\Delta 141-231}$ in organotypic slice cultures of *tg*155 cerebella. We found that the half-life of $PrP_{\Delta 141-231}$ is of about 30 minutes (figure 23), while that of PrP^C using the same set-up is of 8 hours (data communicated by Anna-Maria Callela). However, the antibody used for the measurement of the half-life of the full-length protein (POM1) has a much higher affinity for its ligand than POM12, the

antibody used in the case of $PrP_{\Delta 141\text{-}231}$, which does not allow a direct comparison of the two values.

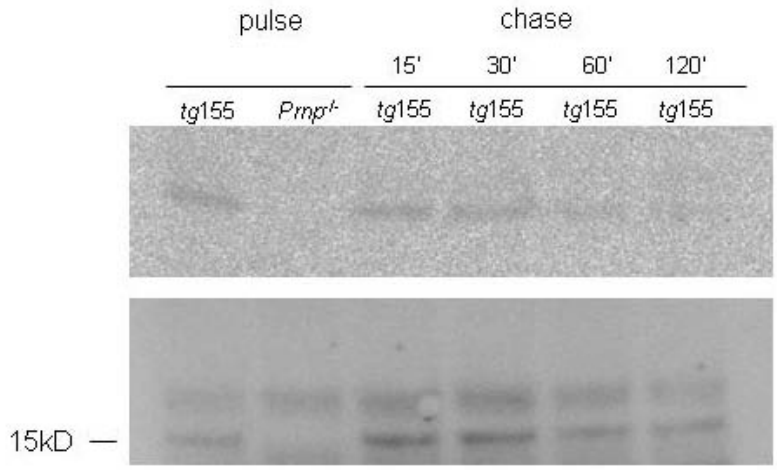


Figure 23

Pulse chase experiment in organotypic slice cultures of mouse cerebella. A Left panel: POM12 immunoprecipitation of cerebellar slice lysate from *tg*155 mice (lane 1, 3, 4, 5 and 6) and $Prnp^{0/0}$ mice (lane 2). Lane 1 and 2: pulse. Lane 3, 4, 5 and 6: chase of 15 minutes, 30 minutes, 1 hour and 2 hours, respectively. Right panel: western blot control of the input. **B** Left panel: immunoprecipitation of cerebellar slice lysate using POM1 from wild-type mice (lane 1, 3, 4, 5 and 6) and $Prnp^{-/-}$ mice (lane 2). Lane 1 and 2: pulse. Lane 3, 4, 5 and 6: chase of 4, 8, 24 and 36 hours, respectively. Right panel: western blot control of the input.

2.2.8 Transgenic mice *tg*155 and *tg*177 have a normal life expectancy and do not develop a spontaneous phenotype

Both transgenic lines expressing $PrP_{\Delta 141\text{-}231}$ developed and reproduced normally and did not present a phenotype in adulthood. Transgenic positive $Prnp^{0/0}$ mice were sacrificed at 20 weeks of age for histological analysis. We did not find any anomalies in the cerebellum (figure 24A), cortex (figure 24B upper panels) and hippocampus (figure 24B lower panels), correlating with absence of phenotype in these mice.

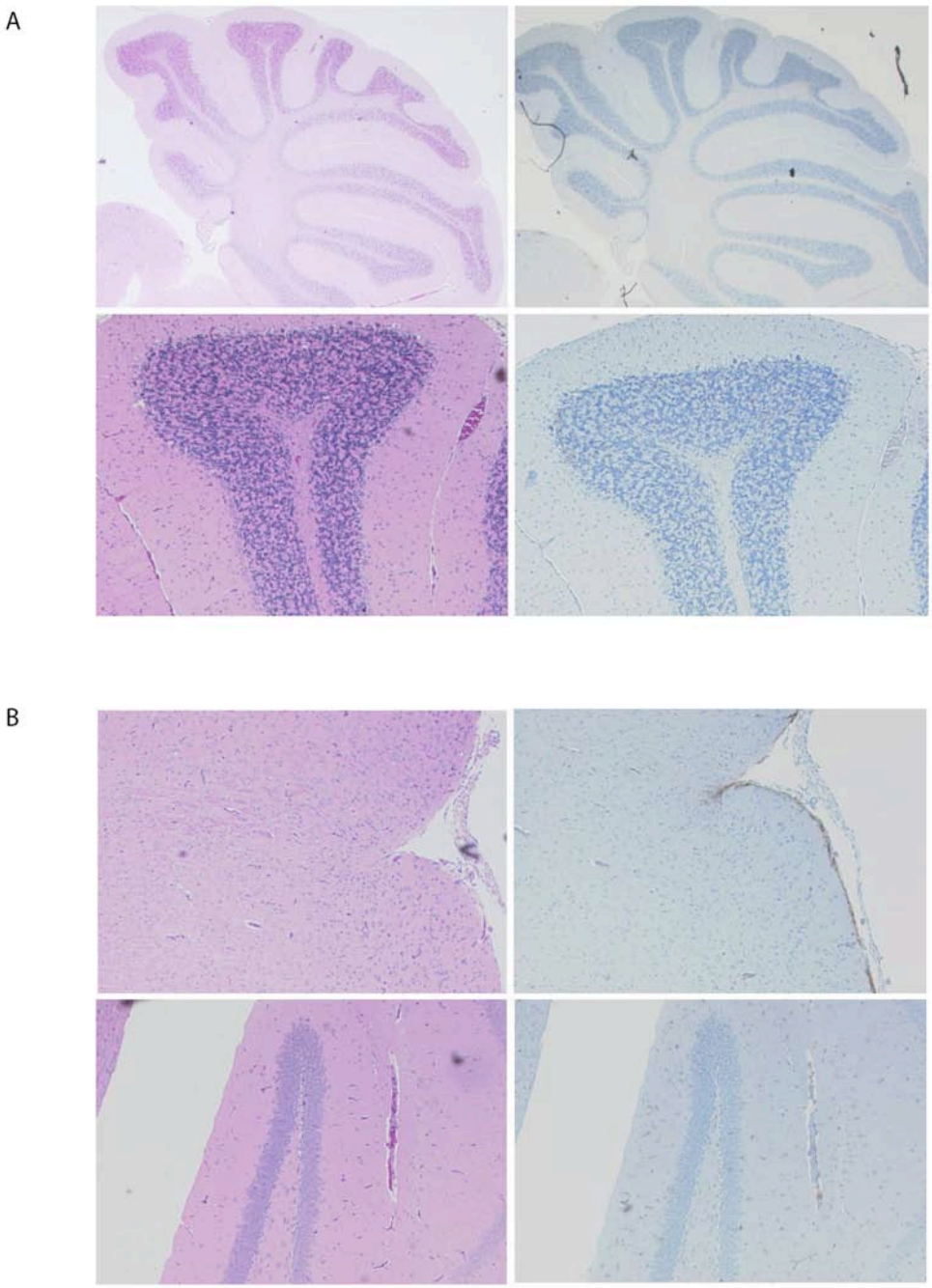


Figure 24

Histological analysis of the CNS of *Prnp*$^{0/0}$ *tg*155 mice. A Cerebellum of 20 weeks old *Prnp*$^{0/0}$ *tg*155 mouse. Left panels, H-E staining. Right panels, GFAP staining. **B** Upper panel: cerebral cortex of 20 weeks old *Prnp*$^{0/0}$ *tg*155 mouse. Left panel, H-E staining. Right panel, GFAP staining. Lower panels : hippocampus of 20 weeks old *Prnp*$^{0/0}$ *tg*155 mouse. Left panel, H-E staining. Right panel, GFAP staining.

2.3 Expression of $PrP_{\Delta 141\text{-}231}$ fails to rescue the phenotype elicited by the expression of $PrP_{\Delta CD}$

2.3.1 Expression level comparison

We compared the brain expression level of $PrP_{\Delta 141\text{-}231}$ in *tg*155 and *tg*177 mice with that of $PrP_{\Delta CD}$ in two transgenic lines, *tg*1046 and *tg*1050 (Baumann, Tolnay et al. 2007). We found that the expression level of $PrP_{\Delta CD}$ in line *tg*1046 is comparable to that of $PrP_{\Delta 141\text{-}231}$ in *tg*155 and *tg*177, while $PrP_{\Delta CD}$ is expressed six times more in *tg*1050 (figure 25).

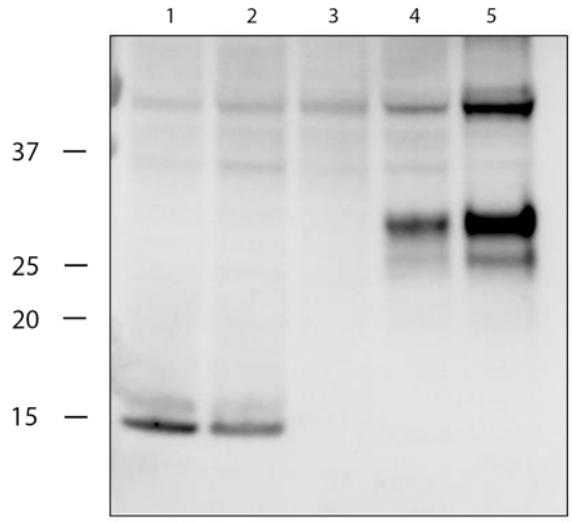


Figure 25

$PrP_{\Delta 141\text{-}231}$ and $PrP_{\Delta CD}$ expression in the brain of transgenic animals. Western blot probed with POM11 (see figure 11). 20ug of brain homogenate per lane. Lane 1: *tg*155; lane 2: *tg*177; lane 3: *Prnp*$^{0/0}$ lane 4: *tg*1046; lane 5: *tg*1050.

2.3.2 Rescue experiment: crossing *tg*155 with *tg*1046 and *tg*1050

In order to assess the ability of $PrP_{\Delta 141-231}$ to convey the function of full-length PrP^C, we crossed *Prnp*$^{0/0}$ *tg*155 mice with *Prnp*$^{0/0}$ *tg*1046 and *Prnp*$^{0/0}$ *tg*1050 mice.

In the absence of a wild-type allele, *Prnp*$^{0/0}$ tg1046 mice die 25 days after birth. The presence of one allele of wild-type PrP^C suffices to rescue completely the phenotype, and *Prnp*$^{+/-}$ *tg*1046 mice have a normal lifespan (Baumann, Tolnay et al. 2007).

The expression level of $PrP_{\Delta CD}$ in *tg*1050 mice is six times higher than in *tg*1046 mice. Accordingly, the neurotoxic phenotype is much stronger and leads to perinatal death of *Prnp*$^{0/0}$ mice. Supraphysiological levels of PrP^C are required to partially rescue the phenotype, and the *tg*1050 line is thus kept on a *tg*a20 (PrP^C overexpressing transgene) hemizygous background, in order to allow survival and reproduction of $PrP_{\Delta CD}$ expressing mice (Baumann, Tolnay et al. 2007).

Double positive *Prnp*$^{0/0}$ mice carrying both the $PrP_{\Delta CD}$ and $PrP_{\Delta 141-231}$ alleles, resulting from either the *tg*155 x *tg*1046 or the *tg*155 x *tg*1050 crossing did not survive longer than single transgenic mice carrying the $PrP_{\Delta CD}$ allele only (figure 26A and B), indicating that $PrP_{\Delta 141-231}$ does not have an impact on the toxic phenotype elicited by $PrP_{\Delta CD}$.

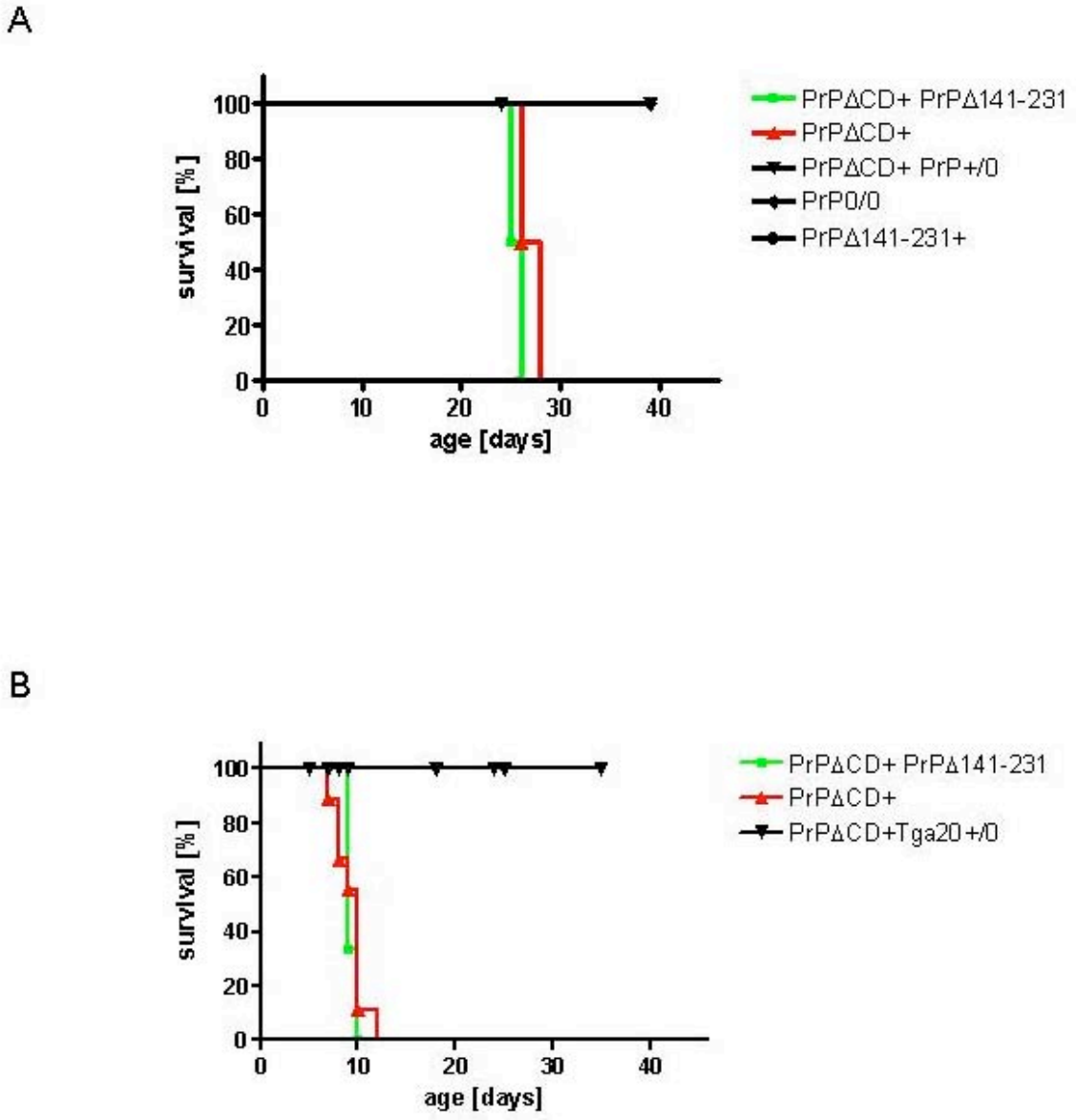


Figure 26

Kaplan-Meyer curve of the offspring resulting from breeding of *tg*155 with two transgenic lines expressing $PrP_{\Delta CD}$. A *tg*155 x *tg*1046. Line tg1046 is kept on a $Prnp^{+/-}$ background. Red triangles are $Prnp^{0/0}$ single positive transgenic mice carrying the $PrP_{\Delta CD}$ allele; green squares are $Prnp^{0/0}$ double positive transgenic mice carrying both the $PrP_{\Delta CD}$ and $PrP_{\Delta 141-231}$ allele; black triangles are $Prnp^{+/-}$ single positive transgenic mice carrying the $PrP_{\Delta CD}$ allele; black diamonds are $Prnp^{0/0}$ mice and black circles are $Prnp^{0/0}$ single positive transgenic mice carrying the $PrP_{\Delta 141-231}$ allele **B** *tg*155 x *tg*1050. The *tg*1050 line is kept on a *tg*a20 hemizygous background to allow survival of the transgene positive offspring. Green squares are $Prnp^{0/0}$ double positive transgenic mice carrying both the $PrP_{\Delta CD}$ and $PrP_{\Delta 141-231}$ allele; red triangles are $Prnp^{0/0}$ single positive transgenic mice carrying the $PrP_{\Delta CD}$ allele; black triangles are $Prnp^{0/0}$ double positive transgenic mice carrying both the $PrP_{\Delta CD}$ and *tg*a20 allele.

3. Discussion

In 1992, the ablation of murine PrP^C was shown to have little if any effect in the CNS, limiting the use of this model to investigate the function of the protein *in vivo* (Bueler, Fischer et al. 1992). A few years later, *Prnp*$^{0/0}$ mice transgenically expressing amino-truncated PrP^C variants were reported to suffer from spontaneous cerebellar neurodegeneration which was rescued by co-expression of full length PrP^C, opening a new avenue to study the physiological function of PrP^C (Shmerling, Hegyi et al. 1998).

The generation of other truncated PrP^C variants bearing smaller deletions in their amino-terminus allowed narrowing down of the region whose disruption leads to neurodegeneration. This region, called the hydrophobic core, is highly conserved amongst all species expressing PrP^C (Baumann, Tolnay et al. 2007) (Li, Christensen et al. 2007).

The central role of the amino-terminus of PrP^C, and in particular of its hydrophobic core in the physiological function of the full-length protein was thus established, and the next logical question to ask was whether the presence of the amino-terminus was not only necessary but also sufficient to convey the function of the full length protein. A first experimental approach to address this question was chosen by Baumann et al, who generated a chimeric protein by substituting the carboxy-terminus of PrP^C with a structural analogue of this domain, Doppel. They showed that, not only was this chimeric protein innocuous when expressed in the CNS, but it was also able to rescue the phenotype elicited by $PrP_{\Delta N}$ (Baumann et al., manuscript in preparation) (figure 8c). A similar construct was recently shown to be able to rescue the toxicity induced by overexpression of Dpl in the CNS (Yoshikawa, Yamaguchi et al. 2008). These findings confirmed the central role played by the amino-terminus of PrP^C in conveying a signal and indicated that while the presence of a structured globular domain might be involved in the function of PrP^C, the amino acid sequence of this domain is not relevant.

To further address the question of the importance of a globular domain in the function of the full-length protein, we generated a redacted version of PrP^C consisting solely of its amino-terminus linked to the GPI anchor signal.

Transgene expression gives rise to a stable protein, both in vitro and in vivo

Our first concern was to assess the stability of $PrP_{\Delta 141\text{-}231}$. A previous study had reported the absence of expression of such a construct *in vitro* (Muramoto, DeArmond et al. 1997). We show that expression both *in vitro* and *in vivo* is possible, and that $PrP_{\Delta 141\text{-}231}$ is located in the same subcellular compartment as wild-type PrP^C.

Two lines of evidence indicate that $PrP_{\Delta 141\text{-}231}$ is not released in the supernatant of transfected cells, but rather located in the lipid raft compartment of the cell, were PrP^C is likely to exert its function. First, the electrophoretic mobility of $PrP_{\Delta 141\text{-}231}$ expressed in a neuronal cell line indicates a higher molecular weight than the secreted version of the same protein, consistent with the presence of a GPI anchor in $PrP_{\Delta 141\text{-}231}$. Second, $PrP_{\Delta 141\text{-}231}$ co-localizes with flotillin, a lipid raft protein, after centrifugation in a step density gradient both *in vitro* and *in vivo*.

Although the RNA levels of $PrP_{\Delta 141\text{-}231}$ in the CNS are high, the protein expression is low, corresponding to 9% of PrP^C expressed in the brain of a wild-type mouse (*tg*155). The half-life of $PrP_{\Delta 141\text{-}231}$ was estimated to 30 minutes, while that of full length PrP^C measured using the same settings but different antibodies was estimated to 8 hours, indicating a reduced stability of $PrP_{\Delta 141\text{-}231}$.

The expression of $PrP_{\Delta 141\text{-}231}$ in vivo does not lead to a phenotype

PrP^C variants expressed transgenically can elicit a toxic phenotype either by interfering with the normal function of PrP^C, as in the case of $PrP_{\Delta N}$ expression, or by accumulating in an inappropriate cellular compartment (toxic gain-of-function), such as for example $cyPrP^C$, a PrP^C variant lacking the signal peptide and accumulating in the cytoplasm (Ma, Wollmann et al. 2002). According to the model proposed by Baumann et al. (Baumann, Tolnay et al. 2007), the expression of $PrP_{\Delta 141\text{-}231}$ is not expected to elicit a phenotype, since it retains the putative functional domain of PrP^C. Transgenic expression of PrP^C variants lacking either the second or the third α-helix were reported to suffer from a disorder resembling a neuronal storage disease. The truncated prion protein variants were not exported to the cell surface and accumulated in the ER (Muramoto, DeArmond et al. 1997). $PrP_{\Delta 141\text{-}231}$ expression did not elicit such a toxic gain-of-function in *tg*155 and *tg*177, although all three helices are absent.

Co-expression of $PrP_{\Delta 141-231}$ does not rescue the ΔCD phenotype

The double positive offspring resulting from the breeding of *tg*155 with two $PrP_{\Delta CD}$ expressing transgenic lines (*tg*1046 and *tg*1050) did not differ in terms of phenotype and survival from the single positive offspring carrying the $PrP_{\Delta CD}$ allele only. Given the high toxicity of $PrP_{\Delta CD}$ in *tg*1050 and the supraphysiological levels of PrP^C required to rescue the phenotype, the results of the *tg*155x*tg*1050 crossing are not surprising. In the case of the lower expresser line *tg*1046, the expression level of $PrP_{\Delta CD}$ corresponds to that of $PrP_{\Delta 141–231}$ in line *tg*155, amounting up to about 10% of PrP^C expression in the CNS of a wild-type mouse (Baumann, Tolnay et al. 2007). This low expression level of $PrP_{\Delta CD}$ is sufficient to induce a strong phenotype, resulting in the death of transgenic mice at 25 days of age, indicating that $PrP_{\Delta CD}$ may have a high affinity for the putative prion protein receptor PrP_R. One allele of *Prnp* is required to rescue the phenotype induced by $PrP_{\Delta CD}$, which represents 5 times more protein than $PrP_{\Delta 141–231}$. Moreover, because $PrP_{\Delta 141–231}$ lacks the globular domain of PrP^C, its interaction with PrP_R might be weaker and require increased levels of $PrP_{\Delta 141–231}$. Therefore, we cannot conclude that the amino-terminus of PrP^C does not convey the function of the full-length protein in the absence of a globular carboxy-terminal domain. Indeed, while a low amount of a toxic mutant ($PrP_{\Delta CD}$) with a potentially high affinity for PrP_R induces a phenotype, the co-expression of an equimolar amount of a protein devoid of the globular domain might not suffice to compete for interaction with PrP_R. The absence of rescue may thus rather be due to the reduced affinity of $PrP_{\Delta 141-231}$ for PrP_R and an increased molar ratio of $PrP_{\Delta 141-231}$ to $PrP_{\Delta CD}$ may be required to compete for interaction with PrP_R.

4. Outlook

PrP^{Sc} treatment with PK removes the octarepeat region without abrogating infectivity (Neary, Caughey et al. 1991). Transgenic mice expressing PrP^{C} deletion mutants lacking the octarepeat region are susceptible to prions (Fischer, Rulicke et al. 1996) (Shmerling, Hegyi et al. 1998). Together, these findings indicate that the octarepeat region of PrP is neither required for infectivity nor for prion propagation. However, PrP^{C} variants with truncations extending further than the octarepeat region, disrupting the charged cluster 2 alone or in conjunction with the hydrophobic core (figure 9), are not convertible into PrP^{Sc}, suggesting that the central domain of PrP^{C} is involved in prion replication (Shmerling, Hegyi et al. 1998). The hydrophobic core is thus a double-edged sword, required both for the physiological function of PrP^{C} and for prion susceptibility. The role of the carboxy-terminus of PrP^{C} in prion propagation is less clear. A patient heterozygous for a *Prnp* mutation consisting of an amber mutation at codon 145 was reported with a GSS syndrome and PrP amyloid plaques, indicating that a PrP^{C} variant lacking the carboxy-domain may be disease-associated (Kitamoto, Iizuka et al. 1993). Transgenic mice expressing PrP mutants lacking helix 2 or helix 3 were shown to be resistant to prion inoculation (Muramoto, DeArmond et al. 1997). However, such mutations led to ER retention of PrP, and the resistance to prions could thus rather be due to the mislocalization of the protein than its inability to convert into a PrP^{Sc} isoform.

We plan to inoculate intracerebrally *Prnp*$^{+/+}$ and *Prnp*$^{+/-}$ *tg*155 mice in order to assess the impact of $PrP_{\Delta 141\text{-}231}$ on prion replication. $PrP_{\Delta 141\text{-}231}$ may be a template for PrP^{Sc}, accelerating the course of the disease, or alternatively act as a dominant negative mutant, binding and sequestrating PrP^{Sc} without converting into a disease associated isoform.

III. The nasal cavity as an entry site for prions

1. Summary and outline of the study

Most cases of naturally occurring animal prion diseases have an infectious aetiology (Sigurdson and Miller 2003). While the epidemic spread of BSE among cattle in the UK during the late 1980's to mid 1990's is attributed to transmission through contaminated feed (Collee and Bradley 1997), the mechanisms of transmission of other prion diseases such as CWD and scrapie remain unknown. Healthy sheep were reported to become infected with prions after gazing in a pasture where scrapie infected sheep had been kept (Poser 2002), and soil has been shown to bind prions and maintain infectivity (Johnson, Phillips et al. 2006), indicating that the environment can be a reservoir for prions.

Many vectors for prion spread have been proposed and experimentally investigated, such as saliva, urine and faeces. CWD was experimentally transmitted to healthy cervids upon oral inoculation with saliva derived from affected animals (Mathiason, Powers et al. 2006); infectivity was detected in the urine of mice inoculated with prions and concomitantly suffering from nephritis (Seeger, Heikenwalder et al. 2005); and faeces of orally inoculated hamsters were shown to be infectious (Safar, Lessard et al. 2008). These experiments indicate that natural secretions/excretions from infected animals are plausible sources of prion contamination of the environment.

The routes of entry of prions into the host remain unclear. Oral, cutaneous (scarified skin) and conjunctival portals of entry have been suggested and proven experimentally (Andreoletti, Berthon et al. 2000) (Sigurdson, Spraker et al. 2001) (Taylor, McConnell et al. 1996) (Scott, Foster et al. 1993).

We hypothesized that the olfactory system, extensively used for various purposes by ruminants, is a site of entry for prions. To model a situation that may occur naturally in animals gazing in an environment contaminated with prions, we inoculated mice by placing mouse-adapted scrapie prions (Rocky Mountain Laboratory (RML) strain) inferior to the nostrils, letting them breathe in the infectivity.

In a first set of experiments, we inoculated transgenic mice overexpressing PrP^C (*tg*a20 mice) (Fischer, Rulicke et al. 1996) to assess a potential prion transmission through this new inoculation route. We show that five out of five mice nasally inoculated with 10^{-2}

RML develop terminal scrapie disease signs 141+/-22 days after inoculation, establishing nasal prion inoculation as a possible route for prion infection.

Upon peripheral prion infection, a replication phase in the lympho-reticular system (LRS) precedes neuroinvasion (Aguzzi and Heikenwalder 2005). Within the lymphoid tissue, follicular dendritic cells (FDCs) were identified as the main prion replication compartment (Weissmann, Raeber et al. 2001). FDCs are radioresistant cells of stromal origin, located in the germinal centres of lymphoid organs. They have a dual function in supporting the formation and maintenance of the lymphoid microarchitecture, and assisting affinity maturation of B lymphocytes by trapping unprocessed antigens. Gene deletion experiments in mice have shown that signalling by both tumor necrosis factor (TNF) and lymphotoxins (LT) is essential for FDC development (Fu, Molina et al. 1997) (Koni, Sacca et al. 1997) (Endres, Alimzhanov et al. 1999). Maintenance of pre-existing FDCs in a differentiated state requires continuous interaction with B cells expressing membrane-bound lymphotoxin-alpha/beta heterotrimers (LT-α/β)) (Gonzalez, Mackay et al. 1998), which signal via the LT-β receptor (LTβR) present on FDCs (Ware, VanArsdale et al. 1995). Mice lacking either B cells (such as in µMT mice, (Kitamura, Roes et al. 1991)) or LTα, LTβ or LTβR, lack mature FDCs and fail to replicate prions in the LRS, preventing the development of cerebral disease (Prinz, Montrasio et al. 2002) (Klein, Frigg et al. 1997). Inhibition of the LTβR pathway in mice by treatment with LTβR–immunoglobulin fusion protein (LTβR-Ig) leads to the dedifferentiation of mature, functional FDCs in spleen and lymph nodes (Gommerman and Browning 2003), significantly impairing peripheral prion pathogenesis and delaying the onset of cerebral disease (Mabbott, Mackay et al. 2000) (Montrasio, Frigg et al. 2000). The chemokine receptor CXCR5 directs lymphocytes towards specific microcompartments (Forster, Mattis et al. 1996). In the absence of CXCR5, the distance between germinal centre-associated FDCs and splenic nerve endings is reduced and prion pathogenesis upon i.p. prion challenge is accelerated (Prinz, Heikenwalder et al. 2003), indicating that the distance between FDCs and nerve endings is critical for neuroinvasion.

FDCs trap antigens either as immune complexes via Fcγ receptors or as C3d/C4b-opsonized antigens through CD21/CD35 receptors. While absence of FcγR has no impact on peripheral prion pathogenesis (Klein, Kaeser et al. 2001) , ablation of CD21/CD35 in the stromal compartment of the LRS reduces susceptibility to peripheral prion inoculation (Zabel, Heikenwalder et al. 2007). Deletion of C3, whose breakdown products bind to CD21/CD35 complement receptors, significantly delays neuroinvasion

upon i.p. inoculation (Klein, Kaeser et al. 2001) (Mabbott, Bruce et al. 2001). C1q is the first component of the classical pathway of the complement system. It can be directly activated by binding to pathogens (Ebenbichler, Thielens et al. 1991), leading to activation of C4, which in turn binds CD21/CD35. Deletion of C1q has a drastic effect on peripheral prion pathogenesis, leading to resistance to low dose peripheral prion inoculation (Klein, Kaeser et al. 2001). Taken together, these data suggest that direct opsonisation of prions may facilitate peripheral prion pathogenesis by enhancing docking to FDCs.

Although FDCs are critical for LRS prion replication, most efficient peripheral prion replication requires both the stromal and haematopoietic compartments of the LRS (Kaeser, Klein et al. 2001). TNFR1$^{-/-}$ are fully susceptible to peripheral administration of prions and infectivity is detected in lymph nodes despite the absence of mature FDCs (Prinz, Montrasio et al. 2002), indicating that other cells than FDCs, probably of haematopoietic origin, can maintain replication of prions to titres that are similar to those of wild-type mice (Prinz, Montrasio et al. 2002). In addition, cells of haematopoietic origin may play an important role in the transport of prions from peripheral entry sites to the lymphoid replicative compartment. For example, upon oral challenge with prions, dendritic cells (DC) can take up prions and act as a cellular bridge between the gut lumen and the LRS (Huang, Farquhar et al. 2002). DC have also been implicated in transport of prions from a cutaneous portal of entry to the draining lymph node (Mohan, Hopkins et al. 2005).

In a second set of experiments that are currently ongoing, we investigate the involvement of the LRS in prion pathogenesis upon nasal inoculation. Owing to the proximity of the nasal mucosa with the central nervous system (Figure 27), we speculate that neuroinvasion occurs directly, without involvement of a replicative step in the LRS. To test this hypothesis, we nasally inoculated different mouse models lacking components known to be crucial for LRS prion replication. The inoculated mice include 1) Mice constitutively lacking mature FDCs: $J_H^{-/-}$ (lack of the J_H gene for antibody heavy chain production, leading to the absence of mature B cells); LT-$\alpha^{-/-}$; LT-$\beta^{-/-}$; LTβR$^{-/-}$ and TNFR1$^{-/-}$ mice; 2) Mice lacking mature FDCs following treatment with LTβR-Ig; 3) CR2-/- and C1q-/- mice lacking CD21/CD35 or C1q respectively; 4) Mice expressing PrP^C on neurons only (NSE-PrP); 5) CXCR5$^{-/-}$ mice with FDCs in close vicinity to splenic nerve endings; 6) CCR7$^{-/-}$ mice lacking secondary lymphoid-tissue chemokine (SLC) receptor, important for the homing of DC to lymph nodes; 7) Appropriate wild-type controls.

If prion transmission upon nasal inoculation were to occur independently of a LRS replication phase, the incubation time in all groups should not differ from that of the appropriate wild-type controls, since none of these mouse models have impaired central prion pathogenesis. If the LRS were to be required for efficient nasal transmission of prions, the panel of transgenic and knock-out mice inoculated here should allow locating the determinant steps of peripheral prion pathogenesis upon nasal inoculation.

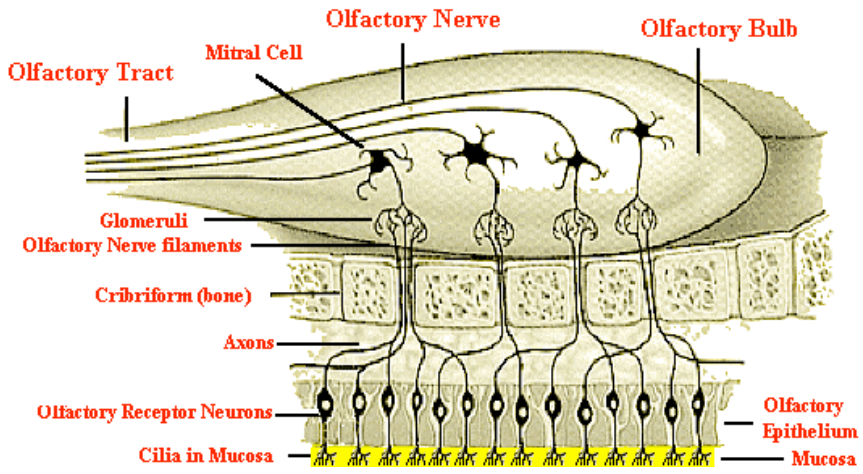


Figure 27

Schematic drawing of the mammalian olfactory system In mammals, the olfactory epithelium lies at the roof of the nasal cavity and contains olfactory sensory neurons whose axons form the olfactory nerve, which belongs to the peripheral nervous system. This nerve crosses the cribriform bone and forms glomeruli within the olfactory bulb. Inside the glomeruli, the axons contact the dendrites of mitral cells. Mitral cells project their axons to brain areas including the piriform cortex, the medial amygdale and the entorhinal cortex.

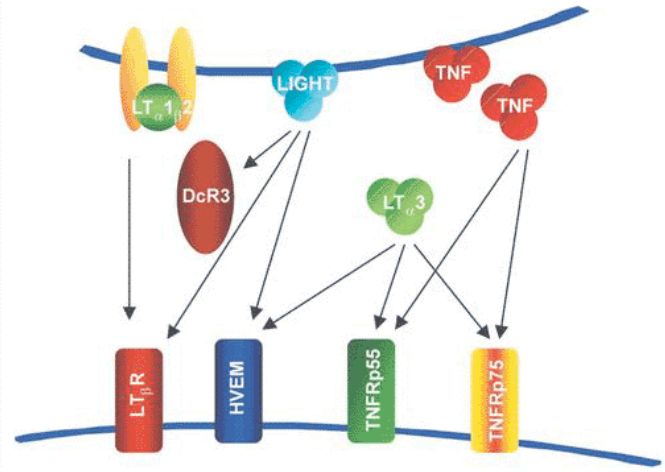


Figure 28

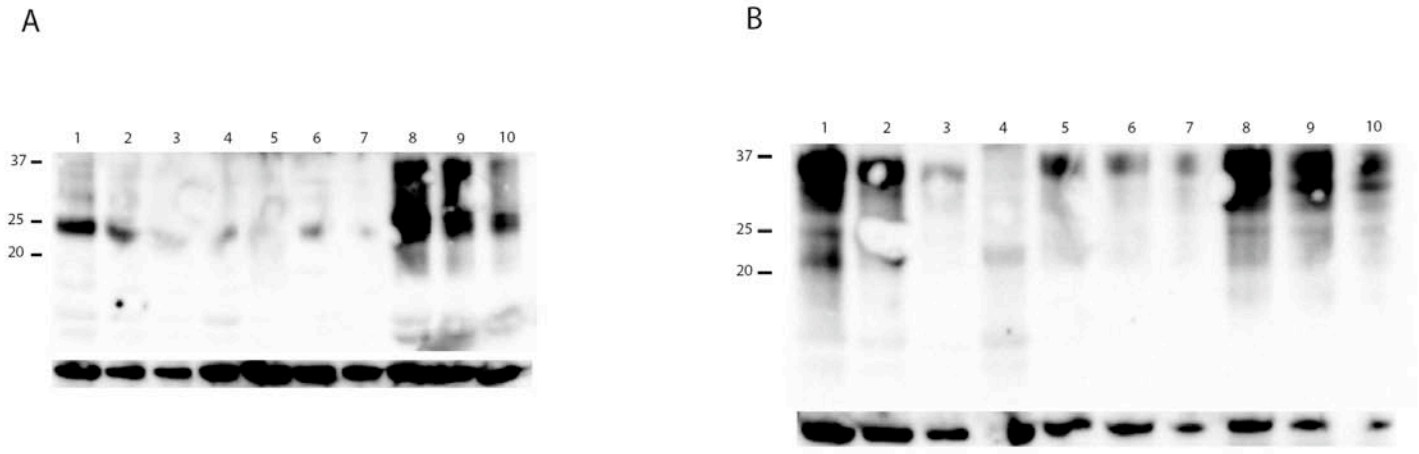
Tumor necrosis factor (TNF), lymphotoxin (LT) and LIGHT ligands, and their receptors. Cell bound and soluble TNF and soluble LTαhomotrimers bind both TNFRp55 (TNFR1) and TNFRp75 (TNFR2). Soluble LTα3 also binds to herpes virus entry mediator (HVEM). Membrane-bound LTα1β2 is only known to bind LTβR, but not the TNFRp55, TNFRp75 or HVEM molecules. LIGHT has been shown to bind to three receptors, HVEM, LTβR and DcR3. LIGHT (TNFSF14), tumor necrosis factor ligand superfamily, member 14; DcR3, decoy receptor 3. From Curr Opin Allergy Clin Immunol 2002

2. Results

2.1 Expression of PrP^C in the respiratory tract and olfactory bulb of wild-type, *tg*a20 and NSE-PrP mice

PrP^C is an absolute prerequisite for prion replication competence, and PrP^C expressing tissue is required for prion spread from peripheral sites to the CNS (Blattler, Brandner et al. 1997) and within the CNS (Brandner, Raeber et al. 1996). While PrP^C expression in the nervous and lymphoid tissue is well characterized, less is known about its expression pattern in the respiratory tract.

We screened PrP^C expression in the respiratory tract and olfactory bulb of transgenic mice overexpressing PrP^C (*tg*a20) or expressing PrP^C exclusively on neurons (NSE-PrP) and wild-type mice. We show that PrP^C is expressed all along the respiratory tract, from the olfactory epithelium to the lungs, as well as in the olfactory bulb (Figure 29).



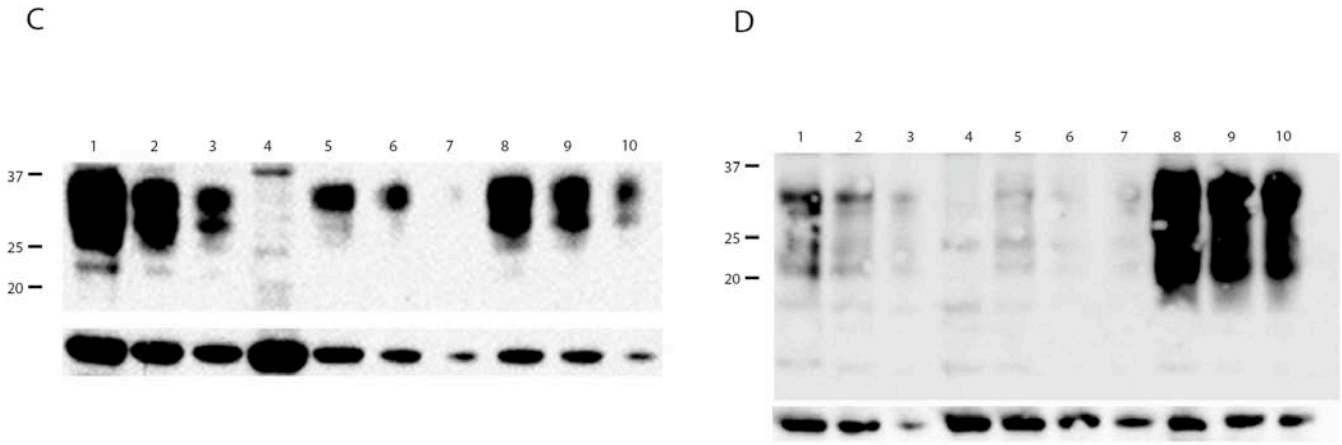


Figure 29

PrP^{C} expression in the respiratory tract of wild-type, NSE-PrP, and *tg*a20 mice. A Trachea. Lane 1 to 3: wild-type mouse; 10ug, 5ug and 2.5ug total protein respectively. Lane 4: *Prnp*$^{0/0}$ mouse. Lane 5 to 7: NSE-PrP mouse; 10ug, 5ug and 2.5ug total protein respectively. Lane 8 to 10: *tg*a20 mouse; 10ug, 5ug and 2.5ug total protein respectively. **B** Lung. Lane 1 to 3: wild-type mouse; 25ug, 12.5ug and 6.25ug total protein respectively. Lane 4: *Prnp*$^{0/0}$ mouse. Lane 5 to 7: NSE-PrP mouse; 25ug, 12.5ug and 6.25ug total protein respectively. Lane 8 to 10: *tg*a20 mouse; 25ug, 12.5ug and 6.25ug total protein respectively. **C** Olfactory bulb. Lane 1 to 3: wild-type mouse; 7.5ug, 3.75ug and 1.9ug total protein respectively. Lane 4: *Prnp*$^{0/0}$ mouse. Lane 5 to 7: NSE-PrP mouse; 7.5ug, 3.75ug and 1.9ug total protein respectively. Lane 8 to 10: *tg*a20 mouse; 7.5ug, 3.75ug and 1.9ug total protein respectively. **D** Respiratory and olfactory epithelium. Lane 1 to 3: wild-type mouse; 10ug, 5ug and 2.5ug total protein respectively. Lane 4: *Prnp*$^{0/0}$ mouse. Lane 5 to 7: NSE-PrP mouse; 10ug, 5ug and 2.5ug total protein respectively. Lane 8 to 10: *tg*a20 mouse; 10ug, 5ug and 2.5ug total protein respectively. PrP^{C} expression gives rise to 3 bands between 37 and 25 kD, corresponding to di-, mono- and unglycosylated species. Upper blot probed with POM1 and lower blot with anti-β actin.

2.2 Nasal inoculation with RML leads to terminal scrapie disease in *tg*a20 mice

To test whether the nasal cavity may be an entry site for prions, we intranasally inoculated nine *tg*a20 mice which overexpress PrP^C under the control of the endogenous PrP^C promoter (Fischer, Rulicke et al. 1996), and four $Prnp^{0/0}$ mice. The inoculation technique is described in the materials and methods section. Briefly, a total of 40ul 10^{-2} mouse adapted sheep prions (RML 5, fifth passage of the RML strain in CD1 mice) were placed under the nostrils of the mice, which breathed it in.

Of the nine *tg*a20 mice, four mice were sacrificed sixty days post-inoculation (dpi) and the remaining five mice were euthanized at end-stage scrapie disease (clinical assessment).

Sixty days post-inoculation, all mice were asymptomatic, and no PK resistant material was detected by conventional western blotting in the respiratory tract, LRS and CNS of the *tg*a20 mice (Figure 30). Approximately eighty days later, the remaining five mice developed end stage scrapie disease. Western blot and histoblot analysis of the LRS and the CNS revealed the presence of PK resistant material in both compartments. The mean survival time was 141 dpi +/- 22. The relatively high standard deviation is most probably attributable to the inoculation route. Indeed, while the same amount of inoculum was given to all mice, the amount that was effectively breathed in by each mouse may vary.

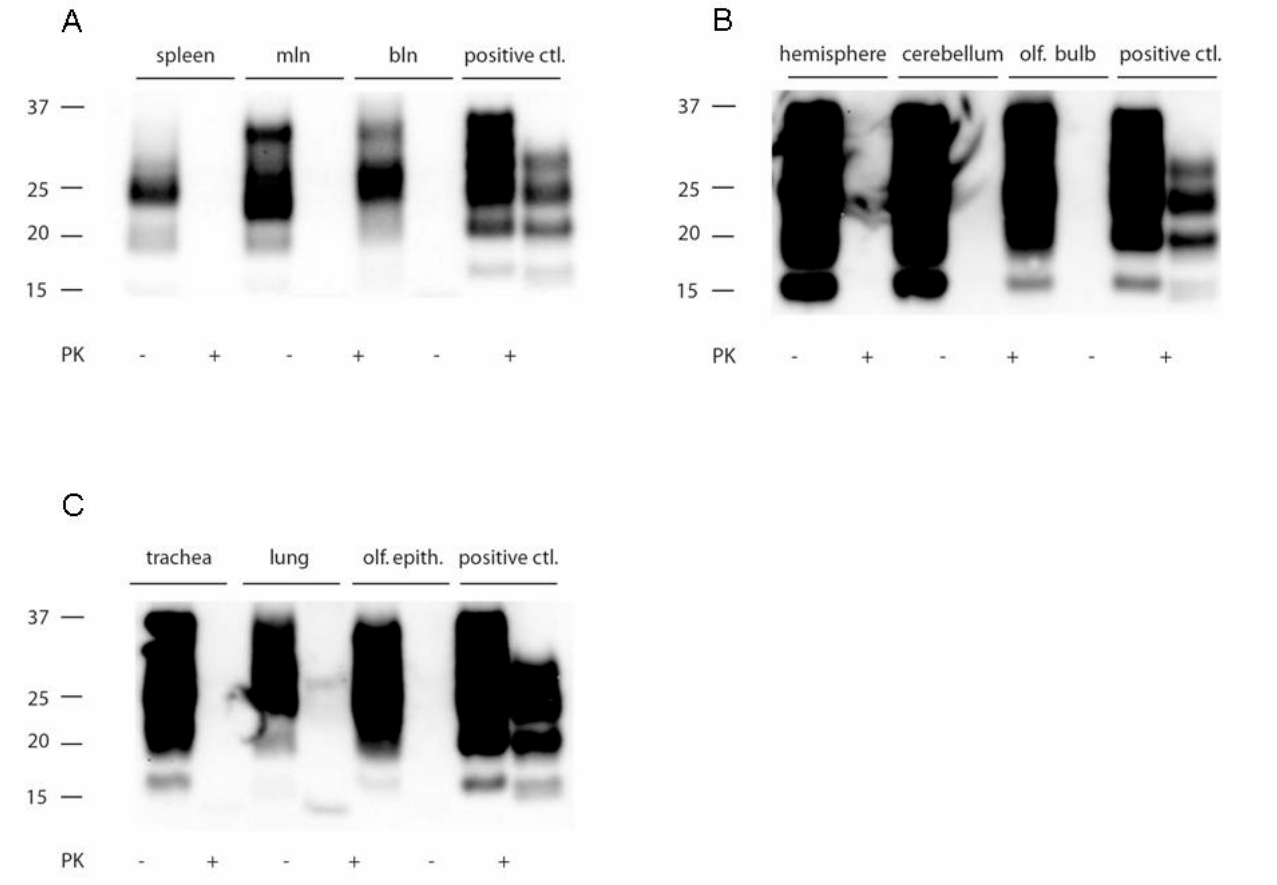


Figure 30

Intranasally inoculated *tg*a20 mouse, 60 days post inoculation. A Western blot of spleen, mesenteric lymph nodes (mln) and bronchal lymph nodes (bln) of a *tg*a20 mouse. 60 days post inoulation, with (PK+) or without (PK-) proteinase K digestion. No PK resistant material is detectable. Positive control: CNS of a terminally sick wild-type mouse. **B** Western blot of the hemispheres, cerebellum and olfactory bulbs of a *tg*a20 mouse 60 days post inoculation, with (PK+) or without (PK-) proteinase K digestion. No PK resistant material is detectable. Positive control: CNS of a terminally sick wild-type mouse. **C** Western blot of trachea, lung, and olfactory epithelium (olf. epith.) of a *tg*a20 mouse, 60 days post inoculation, with (PK+) or without (PK-) proteinase K digestion. No PK resistant material is detectable. Positive control: CNS of a terminally sick wild-type mouse.

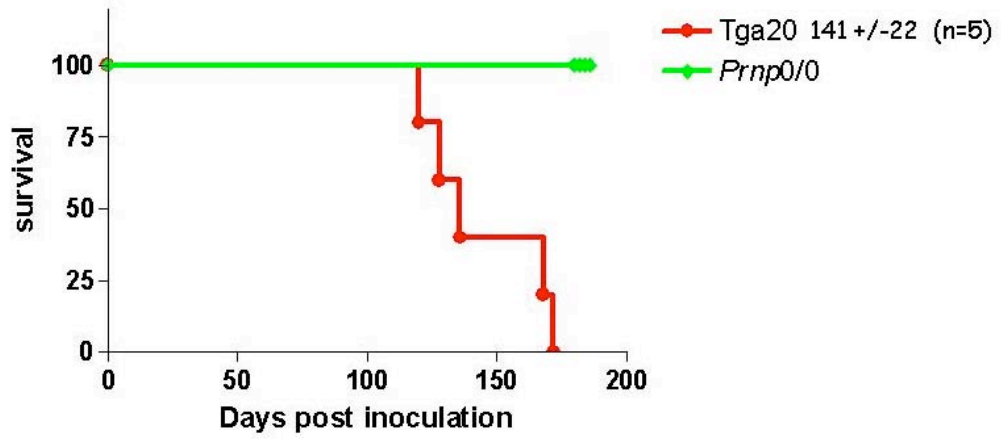


Figure 31

Survival plot of *tg*a20 and Prnp$^{0/0}$ mice intranasally inoculated with 10^{-2} RML5. Five *tg*a20 mice and four Prnp$^{0/0}$ mice were intranasally inoculated with 10^{-2} RML5. All *tg*a20 mice developed terminal scrapie signs and were euthanized. The mean incubation time was 141 dpi +/- 22 days. Red cicles, *tg*a20 mice. Green circles *Prnp*$^{0/0}$ mice.

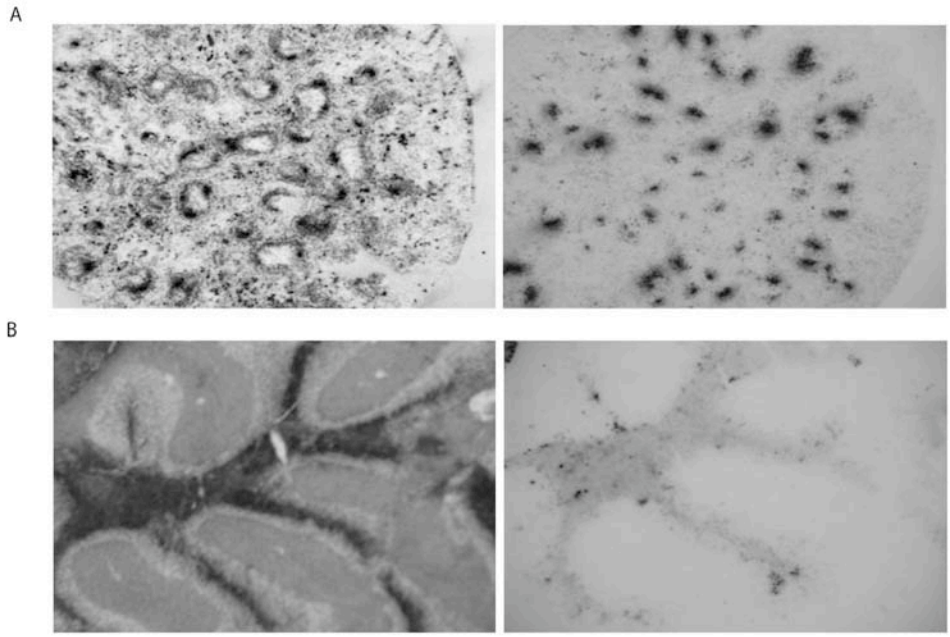


Figure 32

Histoblots of spleen and cerebellum of terminally sick *tg*a20. A Spleen of a terminally sick *tg*a20 mouse inoculated intranasally showing accumulation of PrP^{Sc}. Left panel: no proteinase K (PK) treatment. Right panel: treatment with 100 ug/mL PK. Detection with POM1. **B** Cerebellum of a terminally sick *tg*a20 mouse inoculated intranasally showing accumulation of PrP^{Sc}. Left panel: no proteinase K (PK) treatment. Right panel: treatment with 100 ug/mL PK. Detection with POM1.

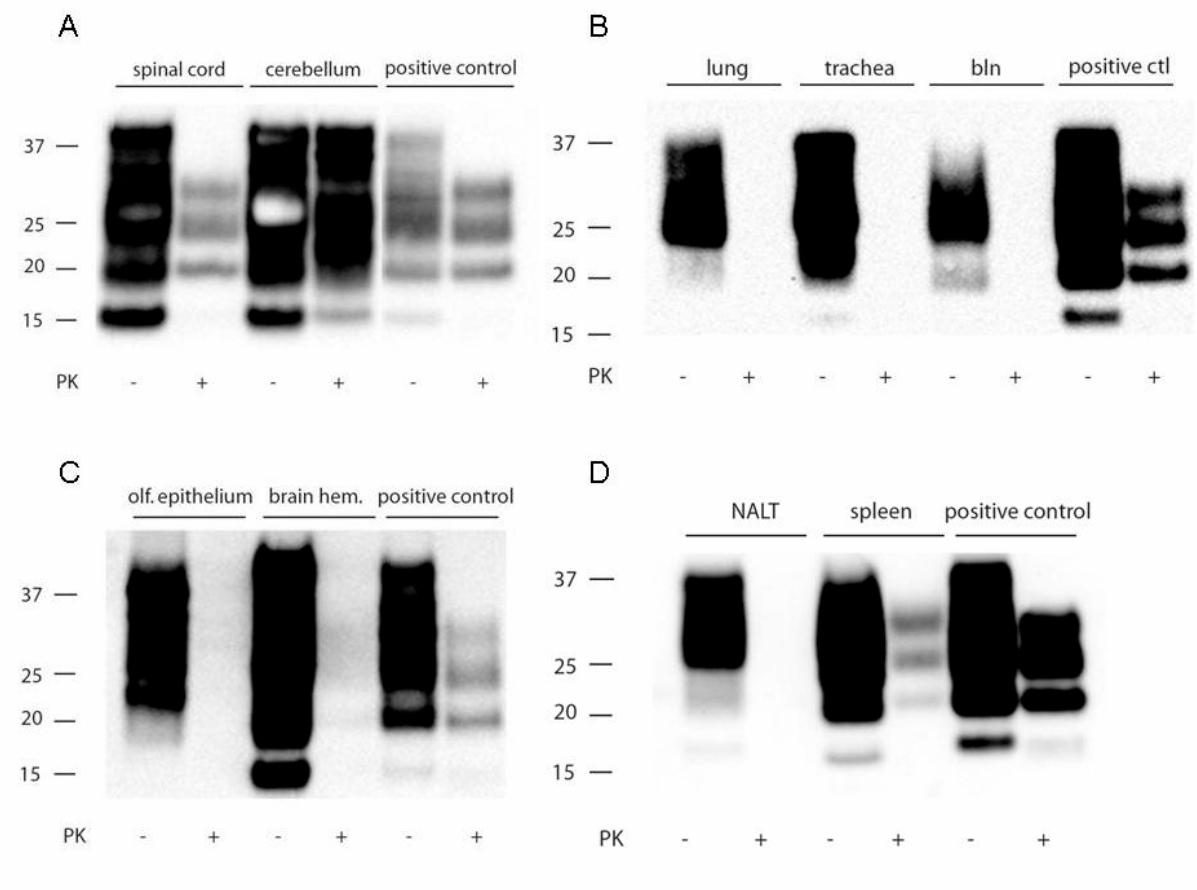


Figure 33

Terminally sick intranasally inoculated *tg*a20 mouse. A Western blot of spinal cord and cerebellum of a terminally sick *tg*a20 mouse with (PK+) or without (PK-) proteinase K digestion. PK resistant material is detectable in both compartments of the CNS. Positive control: CNS of a terminally sick wild-type mouse. **B** Western blot of lung, trachea and bronchial lymph node (bln) of a terminally sick *tg*a20 mouse with (PK+) or without (PK-) proteinase K digestion. PK resistant material is detectable in none of these organs. Positive control: CNS of a terminally sick wild-type mouse. **C** Western blot of the olfactory epithelium and the brain hemispheres of a terminally sick *tg*a20 mouse with (PK+) or without (PK-) proteinase K digestion. PK resistant material is detectable in the hemispheres but not in the olfactory epithelium. Positive control: CNS of a terminally sick wild-type mouse. **D** Western blot of the nasal associated lymphoid tissue (NALT) and the spleen of a terminally sick *tg*a20 mouse with (PK+) or without (PK-) proteinase K digestion. PK resistant material is detectable in the spleen but not in the NALT. Positive control: CNS of a terminally sick wild-type mouse.

2. Discussion and outlook

The natural routes of prion spread among flocks of animals are unknown. The oral portal of entry has been extensively studied, and while this route of transmission is possible, its efficiency is low (Prinz, Huber et al. 2003). At the time we started this study, nothing was known about a potential transmission through the respiratory tract. We hypothesized that the nasal cavity may be an entry site for prions, and assessed this possibility by intranasally inoculating transgenic mice overexpressing PrP^C (*tg*a20). We show that five out of five *tg*a20 mice intranasally inoculated with RML develop terminal scrapie disease, identifying the nasal cavity as a possible entry site for prions.

The determination of the efficiency of intranasal prion infection requires endpoint titration of RML and determination of the LD_{50} (lethal dose for 50% of inoculated mice) (Prusiner, Cochran et al. 1982), followed by comparison of the infectivity titres upon intranasal inoculation with those of established infection routes such as oral, intraperitoneal (i.p.) and intracerebral (i.c.) routes. Although these titration experiments have not been done yet, the nasal route of infection appears to be more efficient than the oral route. Indeed, the same inoculum administrated orally fails to elicit disease in *tg*a20 mice, while inducing 100% lethality upon intranasal inoculation (results communicated by Mathias Heikenwälder).

Given the anatomical proximity of the respiratory and digestive tracts, it is technically difficult to exclude that part of the intranasal inoculum is not swallowed by the mouse. However, because efficient prion transmission through the oral route requires higher doses than the one used in this study, we can argue that the respiratory tract participates to a great extent in the transmission of prions upon intranasal inoculation.

At sixty days post inoculation, PrP^{Sc} was neither detected in the LRS nor in the CNS of intranasally inoculated *tg*a20. This stands in contrast with what is known for other peripheral routes of inoculation (oral and i.p.), where PrP^{Sc} levels rise rapidly in the LRS of both wild-type and *tg*a20 mice, maintaining a plateau level throughout the course of the disease. Recently, a publication reported the high efficiency of prion transmission through the nasal cavity in hamsters using an inoculation protocol similar to ours (Kincaid and Bartz 2007). They further report the presence of PrP^{Sc} in the LRS four weeks after nasal inoculation. Our preliminary results may indicate that, similarly to what

is observed in *tg*a20 mice inoculated in the sciatic nerve or in the footpad (Glatzel and Aguzzi 2000), PrP^{C} overexpression bypasses the need for a replication phase in LRS upon nasal route in *tg*a20 mice. The study of prion transmission upon nasal inoculation in wild-type mice (ongoing) will allow us to address this question and compare our results with those reported for hamsters.

At end-stage disease, PrP^{Sc} was present in both the LRS and the CNS. These findings do not allow conclusions on the requirement the LRS for a replication step before neuroinvasion. Indeed, upon i.c. prion inoculation of wild-type and *tg*a20 mice, PrP^{Sc} is detectable in the LRS at end stage disease, although a replication step in the LRS is not required for CNS disease. The requirement of the LRS for prion pathogenesis upon nasal inoculation will be best addressed in the second and ongoing part of this project.

The identification of novel pathways of prion transmission may lead to the reevaluation of tissues and organs that were previously assumed to be prion free. It may also lead to the identification of situations of potential iatrogenic transmission of prion diseases. PrP^{Sc} has been detected upon autopsy in the olfactory epithelium of patients suffering from sCJD (Zanusso, Ferrari et al. 2003). While there is no epidemiological evidence for direct transmission of prions from individual to individual (Linsell, Cousens et al. 2004), the nasal cavity may be a route of indirect prion transmission through contaminated respiratory devices. However, it remains to be determined whether sCJD patients carry PrP^{Sc} in their olfactory mucosa at earlier time-points of the disease, when medical procedures may be done in the absence of the diagnosis of sCJD.

V. Material and methods

1. RNA extraction and cDNA synthesis

Total RNA from 8 weeks old mouse brains was isolated using 1mL Trizol (invitrogen) per 50mg of tissue. 1ug RNA was used for cDNA synthesis. Prior to cDNA synthesis, contaminating genomic DNA was removed. cDNA synthesis was done with QuantiTect Reverse transcription Kit (Qiagen) according to the manufacturer's instructions.

2. Construction of the $PrP_{\Delta 141-231}$ transgene

Total mouse brain cDNA was amplified using the primer set SY6 and SY7 which introduce BamHI and SalI cloning sites at the 3' and 5' ends of the ORF of PrP^C .
PrP^C was cloned into the cloning vector pBSK (pBSK-PrP). This plasmid served as a template for the following cloning steps.
An XbaI cloning site was introduced by PCR in the PrP ORF just before the GPI signal sequence (amino acid 231). The primer sets used were SY6/SY6a and SY7a/SY7.
An amino-terminal insert was amplified from pBSK-PrP using the primer set SY6/SY8 which introduces BamHI and XbaI cloning sites before the start codon and after amino acid 140, respectively.
pBSK-PrP and the amino-terminal insert were digested with BamHI and XbaI, and the open vector and the insert were ligated, to generate a construct consisting of the first 140 amino acids of PrP^C directly linked to the GPI signal ($PrP_{\Delta 141-231}$).
The transgene was then transferred to the expression vector pBMN, by digestion with BamHI and SalI followed by ligation.
Primers:
SY6 (forward): 5'-CGCGGATCCAATTTAGGAGAGCCAAGCAGA-3'
SY6a (reverse): 5'-CGCTCTAGAACGTCGCCCGTCGTAATAGGC-3'
SY7a (forward): 5'-CGCTCTAGAGACGGGAGAAGATCCAGCAGC-3'
SY7 (reverse): 5'-ACGCGTCGACCACGAGAATGCGAAGGAACA-3'
SY8 (reverse): 5'-ACGCTCTAGACCAGTCGTTGCCAAAATG-3'

3. Transduction of cells

Retroviral particles were generated with the Phoenix Retrovirus Expression System (Orbigen), following the manufacturer's instructions. The adequate cell line (N2a or Hpl) was transduced, and cells were harvested and processed for downstream experiments (Western blotting or flotation assays).

4. Immunoprecipitation and western blotting

Paramagnetic beads (tosylactivated M280 Dynabeads, Dynal) were conjugated with POM12 or mouse IgG1 isotype control antibody according to the manufacturer's manual (3 ug antibody per 10^7 beads). Protein concentrations of 10 % brain homogenates prepared in RIPA buffer (50 mM Tris-HCl pH8, 150 mM NaCl, 0.1% SDS, 1% NP-40) were determined by BCA (bicinchoninic acid) Protein Assays (Pierce), according to the manufacturer's instructions. The homogenates were incubated with the beads, washed and resuspended in RIPA containing loading buffer (NUPAGE LDS Sample Buffer, Invitrogen). And subjected to Western blotting. Blots were blocked with 5% Topblock (Sigma) and incubated with the appropriate POM antibody (1:10'000, Polymenidou et al 2005) in 1% Topbloc. For detection, rabbit anti-mouse IgG1-HRP (Zymed, 1:10'000) was used . After adding substrate (SuperSignal West Dura, Pierce), blots were analyzed with a FUJI LAS 3000 imaging system.

5. Flotation assays

Flotation of detergent insoluble complexes was performed as described (Naslavsky *et al*., 1997). Briefly, brain homogenates were extracted for 2 h on ice in cold lysis buffer (150 mM NaCl, 25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Triton X-100); total protein: 1mg in 1 mL. Extracts were mixed with two volumes (2 ml) of 60% Optiprep (Nycomed) to reach a final concentration of 40%. All lysates were loaded at the bottom of Beckman ultracentrifuge tubes. A 5–30% Optiprep® step gradient in TNE (150 mM NaCl, 25 mM Tris–HCl, pH 7.5, 5 mM EDTA) was then overlaid onto the lysate (8.4 ml of 30%

Optiprep® and 3.6 ml of 5% Optiprep®). Tubes were centrifuged for 24 h at 4°C in a TLS55 Beckman rotor at 100 000 *g*. Fractions (1 ml) were collected from the top of the tube and processed for immunoblotting.

6. Quantitative Real Time PCR (qPCR) analysis and primers

Quantitative real-time PCR was performed using SYBR Green Master Mix (Qiagen) on a 7900HT Fast Real-Time PCR System (Applied Biosystems) using default cycling conditions. Expression levels were normalized using *Gapdh*. The *Prnp* primers were design to amplify the amino-terminal domain of PrP^{C} only, so that the amplification product of *Prnp* and the $PrP_{\Delta 141\text{-}231}$ transgene would be the same.

Primers:

Gapdh forward primer: 5'-CCACCCCAGCAAGGAGACT-3'

Gapdh reverse primer: 5'-GAAATTGTGAGGGAGATGCT-3'

Prnp forward primer: 5'-ATGGCGAACCTTGGCTAC-3'

Prnp reverse primer: 5'-AAAATGGATCATGGGCCTG-3'

7. Generation of the transgenic mouse

The phg plasmid containing the $PrP_{\Delta 141\text{-}231}$ ORF was propagated in *Escherichia coli* XL1 blue, the minigene was excised with *Not*I and *Sal*I (see figure 16 B), processed as described (Fischer et al, 1996), and injected into fertilized $Prnp^{+/+}$ oocytes (B6D2F1/Crl) by standard procedures (Rulicke, 2004). The transgene positive founders were then crossed with $Prnp^{0/0}$ ZHI mice in order to obtain transgene positive mice on a $Prnp^{0/0}$ background. Transgenes were identified by PCR using the exon 2 forward primer pE2 and the non-coding region at 3' of exon 3 reverse primer 3'NC . The fragment size of the transgene was 647bp. In order to outbreed the *Prnp*+ allele, PCR analysis was carried out using primers P10 (forward primer, *Prnp* exon 3), 3'NC (reverse primer), and P3 (reverse primer, neoR gene); P10 and 3'NC gave a 542 bp signal for the $Prnp^{+}$ allele and P3 and 3'NC gave a 362 bp product for the $Prnp^{0}$ allele. In the PCR, the transgene was also detected as a 269 bp product.

Primers:

pE2: 5'-CAA CCG AGC TGA AGC ATT CTG CCT-3'

3'NC: 5'-CCC TCC CCC AGC CTA GAC CAC GA-3'

P10: 5'-GTA CCC ATA ATC AGT GGA ACA AGC CCA GC-3'

P3: 5'-ATT CGC AGC GCA TCG CCT TCT ATC GCC-3'

8. Southern blot analysis

Mouse tail biopsies were digested in lysis buffer (10mM Tris-HCl pH 8, 5mM EDTA pH 8, 1% SDS, 300 mM NaAC pH 7.6 and 100 µg/ml Proteinase K) overnight at 55°C under constant agitation. Purified genomic DNA (50 µg) was digested overnight (EcoRI, 100 U) and separated on 0.7% agarose gels. Completeness of the restriction digest and DNA separation was checked by ethidium bromide staining. Genomic DNA was transferred by overnight blotting in 20xSSC (3M NaCl, 0.3M Na3Citrate, pH 7.4) onto a positively charged biodyne B membrane . The blot was marked, washed for 5 min in 2 × SSC, UV-crosslinked and prehybridized according to Church and Gilbert (1984). After hybridization (overnight at 65°C) with a radioactively labeled probe ($PrP_{\Delta 14\text{-}231}$ ORF), the blot was washed and membranes were subjected to autoradiography using a PhosphoImager (Molecular Dynamics). Images were scanned and signals were quantified with Aida 2.41 imaging software. For probe labelling, template DNA ($PrP_{\Delta 14\text{-}231}$ ORF, 50 ng) was diluted in sterile water and random primers (nonamers or hexamers) were added (Prime-IT® II; Random Primer labelling kit cat# 300385; Stratagene). After gentle mixing, incubation was performed at 95°C for 5 min. Brief centrifugation was followed by 3 min incubation on ice. This was followed by addition of 5 × primer buffer (−dCTP), radioactively labeled [α−32]P-dCTP (corresponding to 25 µCi; Amersham Biosciences), and Klenow 5 U/µl (Prime-IT® II; Random Primer labelling kit cat# 300385). For primer elongation, the probe was incubated at 37°C for 75 min. For purification, the QIAquick PCR purification kit was used according to the manufacturer's manual (Quiagen).

9. Pulse-chase experiment

Cerebellar slice cultures were prepared as described (Falsig and Aguzzi 2008). Four inserts containing eight cerebellar slices were prepared using *tg*155 mice and one insert was prepared using *Prnp*$^{0/0}$ mice as control for the specificity of the immunoprecipitation. The slices were kept in culture for four weeks prior to the pulse-chase experiment. The inserts containing the slices were washed twice with phosphate-buffered saline (PBS) and starved for 1 hour in RPMI medium without methionine/cysteine containing 1% foetal calf serum. Labeling was carried out by adding 400 µCi/ml [35S]Met/Cys to the medium for 30 minutes. After incubation, the slices were washed twice in cold PBS and harvested with RIPA buffer (pulse slices) (50 mM Tris-HCl pH8, 150 mM NaCl, 0.1% SDS, 1% NP-40) or incubated at 37 °C for 15 minutes, 30 minutes, 60 minutes and 120 minutes in complete culture medium (chase slices). After appropriate chase times, the slices were washed with PBS and lysed with cold RIPA buffer and kept on ice for 30 minutes. Cell debris were removed by centrifugation for 10 minutes at 18,000 × g. The supernatents were immunoprecipitated using POM12 coupled Dynabeads as described in section 4. The material released from the beads by boiling was loaded on two SDS-PAGE. One gel was blotted on a nitrocellulose membrane and the membrane probed with POM11. The second gel was desiccated and subjected to autoradiography. The signals were quantified by sing a PhosphoImager (Molecular Dynamics).

10. Morphological analysis

Brains were removed and fixed in 4% formaldehyde in PBS, pH 7.5, paraffin embedded, and cut into 2–4 µm sections. Sections were stained with hematoxylin and eosin (H&E) and GFAP (glial fibrillary acidic protein; activated astrocytes).

11. Prion inoculation of mice

Mice were inoculated with a 1% homogenate of heat- and sarcosyl-treated brain prepared from mice infected with the Rocky Mountain Laboratory (RML) scrapie strain, 5th passage in CD1 mice (RML5). Mice were anaesthetized with Xylazin/Ketamin and

held horizontally during the entire intranasal inoculation procedure. Ten µl were deposited under each nostrils, and the mouse was allowed to breath in the inoculum. A pause of 10 minutes was made before the procedure was repeated once, in order to inoculate a total of forty ul. Mice were monitored every third day, and scrapie was diagnosed according to standard clinical signs.

12. Western blot of infected mice

Homogenates (10%, w/v) of the appropriate tissues were prepared as described (Bueler, Aguzzi et al. 1993) and, where indicated, digested with 20 µg/ml proteinase K for 30 min at 37 °C. The samples were then electrophoresed through 12%–SDS polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were probed with POM1 (1:10'000, Polymenidou et al 2005) in 1% Topbloc to mouse PrP^C, and with anti-β-actin to mouse β-actin. The blots were developed by enhanced chemiluminescence (Amersham).

13. Histoblot

The histoblot technique was performed according to protocols of Taraboulos et al. (Taraboulos, Jendroska et al. 1992). Frozen sections of 8 µm thickness were mounted on uncoated glass slides and immediately pressed on a nitrocellulose membrane wetted in lysis buffer. Membranes were air-dried for at least 24 h. For detection, they were rehydrated in TBST, and limited proteolysis was performed using PK concentrations of 50 and 100 µg/ml at 37 °C for 4 h. Blots were then denatured in 3 M guanidinium thiocyanate for 10 min and blocked for 1 h in 5% non-fat milk serum. Incubation with primary antibody POM1 was carried out at a dilution of 1:2000 in 1% non-fat milk serum at room temperature for 1 h. Detection was accomplished with an alkaline phosphatase-conjugated goat anti-mouse antibody at a concentration of 1:2000. Visualization was achieved with nitro blue tetrazolium and bromo-chloro-indolyl phosphate according to the protocols of the supplier.

Aguzzi, A. (2003). "Prions and the immune system: a journey through gut, spleen, and nerves." Adv Immunol **81**: 123-71.

Aguzzi, A., F. Baumann, et al. (2008). "The Prion's Elusive Reason for Being." Annu Rev Neurosci **31**: 439-77.

Aguzzi, A. and M. Heikenwalder (2005). "Prions, cytokines, and chemokines: a meeting in lymphoid organs." Immunity **22**(2): 145-54.

Aguzzi, A., M. Heikenwalder, et al. (2007). "Insights into prion strains and neurotoxicity." Nat Rev Mol Cell Biol **8**(7): 552-61.

Aguzzi, A., C. Sigurdson, et al. (2007). "Molecular Mechanisms of Prion Pathogenesis." Annu Rev Pathol.

Aguzzi, A. and C. J. Sigurdson (2004). "Antiprion immunotherapy: to suppress or to stimulate?" Nat Rev Immunol **4**(9): 725-36.

Alper, T., W. A. Cramp, et al. (1967). "Does the agent of scrapie replicate without nucleic acid?" Nature **214**(5090): 764-6.

Alper, T., D. A. Haig, et al. (1966). "The exceptionally small size of the scrapie agent." Biochem Biophys Res Commun **22**(3): 278-84.

Alper, T., D. A. Haig, et al. (1978). "The scrapie agent: evidence against its dependence for replication on intrinsic nucleic acid." J Gen Virol **41**(3): 503-16.

Andreoletti, O., P. Berthon, et al. (2000). "Early accumulation of PrP(Sc) in gut-associated lymphoid and nervous tissues of susceptible sheep from a Romanov flock with natural scrapie." J Gen Virol **81**(Pt 12): 3115-26.

Aucouturier, P., R. J. Kascsak, et al. (1999). "Biochemical and conformational variability of human prion strains in sporadic Creutzfeldt-Jakob disease." Neurosci Lett **274**(1): 33-6.

Basler, K., B. Oesch, et al. (1986). "Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene." Cell **46**(3): 417-28.

Baumann, F. (unpublished results).

Baumann, F., M. Tolnay, et al. (2007). "Lethal recessive myelin toxicity of prion protein lacking its central domain." Embo J **26**(2): 538-47.

Beekes, M., E. Baldauf, et al. (1996). "Sequential appearance and accumulation of pathognomonic markers in the central nervous system of hamsters orally infected with scrapie." J Gen Virol **77 (Pt 8)**: 1925-34.

Behrens, A. and A. Aguzzi (2002). "Small is not beautiful: antagonizing functions for the prion protein PrP(C) and its homologue Dpl." Trends Neurosci **25**(3): 150-4.

Behrens, A., N. Genoud, et al. (2002). "Absence of the prion protein homologue Doppel causes male sterility." Embo J **21**(14): 3652-8.

Bellon, A., W. Seyfert-Brandt, et al. (2003). "Improved conformation-dependent immunoassay: suitability for human prion detection with enhanced sensitivity." J Gen Virol **84**(Pt 7): 1921-5.

Bendheim, P. E., H. R. Brown, et al. (1992). "Nearly ubiquitous tissue distribution of the scrapie agent precursor protein." Neurology **42**(1): 149-56.

Berndt, R. (1954). "Reaction to contact in the Eastern Highlands of New Guinea." Oceania **24**: 190-206.

Bessen, R. A. and R. F. Marsh (1992). "Biochemical and physical properties of the prion protein from two strains of the transmissible mink encephalopathy agent." J Virol **66**(4): 2096-101.

Bessen, R. A. and R. F. Marsh (1994). "Distinct PrP properties suggest the molecular basis of strain variation in transmissible mink encephalopathy." J Virol **68**(12): 7859-68.

Blattler, T., S. Brandner, et al. (1997). "PrP-expressing tissue required for transfer of scrapie infectivity from spleen to brain." Nature **389**(6646): 69-73.

Bolton, D. C., M. P. McKinley, et al. (1982). "Identification of a protein that purifies with the scrapie prion." Science **218**(4579): 1309-11.

Bolton, D. C., R. K. Meyer, et al. (1985). "Scrapie PrP 27-30 is a sialoglycoprotein." J Virol **53**(2): 596-606.

Borchelt, D. R., M. Scott, et al. (1990). "Scrapie and cellular prion proteins differ in their kinetics of synthesis and topology in cultured cells." J Cell Biol **110**(3): 743-52.

Bounhar, Y., Y. Zhang, et al. (2001). "Prion protein protects human neurons against Bax-mediated apoptosis." J Biol Chem **276**(42): 39145-9.

Bradley, R. (2001). "Bovine spongiform encephalopathy and its relationship to the new variant form of Creutzfeldt-Jakob disease. An account of bovine spongiform encephalopathy, its cause, the clinical signs and epidemiology including the transmissibility of prion diseases with special reference to the relationship between bovine spongiform encephalopathy and the variant form of Creutzfeldt-Jakob disease." Contrib Microbiol **7**: 105-44.

Brandner, S., S. Isenmann, et al. (1996). "Normal host prion protein necessary for scrapie-induced neurotoxicity." Nature **379**(6563): 339-43.

Brandner, S., A. Raeber, et al. (1996). "Normal host prion protein (PrPC) is required for scrapie spread within the central nervous system." Proc Natl Acad Sci U S A **93**(23): 13148-51.

Brown, D. R., K. Qin, et al. (1997). "The cellular prion protein binds copper in vivo." Nature **390**(6661): 684-7.

Brown, D. R., B. Schmidt, et al. (1997). "Effects of oxidative stress on prion protein expression in PC12 cells." Int J Dev Neurosci **15**(8): 961-72.

Brown, D. R., W. J. Schulz-Schaeffer, et al. (1997). "Prion protein-deficient cells show altered response to oxidative stress due to decreased SOD-1 activity." Exp Neurol **146**(1): 104-12.

Brown, D. R., B. S. Wong, et al. (1999). "Normal prion protein has an activity like that of superoxide dismutase." Biochem J **344 Pt 1**: 1-5.

Brown, P. and R. Bradley (1998). "1755 and all that: a historical primer of transmissible spongiform encephalopathy." Bmj **317**(7174): 1688-92.

Brown, P., F. Cathala, et al. (1987). "The epidemiology of Creutzfeldt-Jakob disease: conclusion of a 15-year investigation in France and review of the world literature." Neurology **37**(6): 895-904.

Bruce, M. E., K. L. Brown, et al. (2000). "Follicular dendritic cells in TSE pathogenesis." Immunol Today **21**(9): 442-6.

Buddi, R., B. Lin, et al. (2002). "Evidence of oxidative stress in human corneal diseases." J Histochem Cytochem **50**(3): 341-51.

Budka, H. (2003). "Neuropathology of prion diseases." Br Med Bull **66**: 121-30.

Budka, H., A. Aguzzi, et al. (1995). "Neuropathological diagnostic criteria for Creutzfeldt-Jakob disease (CJD) and other human spongiform encephalopathies (prion diseases)." Brain Pathol **5**(4): 459-66.

Bueler, H., A. Aguzzi, et al. (1993). "Mice devoid of PrP are resistant to scrapie." Cell **73**(7): 1339-47.

Bueler, H., M. Fischer, et al. (1992). "Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein." Nature **356**(6370): 577-82.

Campana, V., D. Sarnataro, et al. (2006). "Detergent-resistant membrane domains but not the proteasome are involved in the misfolding of a PrP mutant retained in the endoplasmic reticulum." J Cell Sci **119**(Pt 3): 433-42.

Cancellotti, E., F. Wiseman, et al. (2005). "Altered glycosylated PrP proteins can have different neuronal trafficking in brain but do not acquire scrapie-like properties." J Biol Chem **280**(52): 42909-18.

Caughey, B. (2003). "Prion protein conversions: insight into mechanisms, TSE transmission barriers and strains." Br Med Bull **66**: 109-20.

Caughey, B. W., A. Dong, et al. (1991). "Secondary structure analysis of the scrapie-associated protein PrP 27-30 in water by infrared spectroscopy." Biochemistry **30**(31): 7672-80.

Chatelain, J., F. Cathala, et al. (1981). "Epidemiologic comparisons between Creutzfeldt-Jakob disease and scrapie in France during the 12-year period 1968-1979." J Neurol Sci **51**(3): 329-37.

Chen, S. G., D. B. Teplow, et al. (1995). "Truncated forms of the human prion protein in normal brain and in prion diseases." J Biol Chem **270**(32): 19173-80.

Chesebro, B., M. Trifilo, et al. (2005). "Anchorless prion protein results in infectious amyloid disease without clinical scrapie." Science **308**(5727): 1435-9.

Chiesa, R., B. Drisaldi, et al. (2000). "Accumulation of protease-resistant prion protein (PrP) and apoptosis of cerebellar granule cells in transgenic mice expressing a PrP insertional mutation." Proc Natl Acad Sci U S A **97**(10): 5574-9.

Chiesa, R., P. Piccardo, et al. (2003). "Molecular distinction between pathogenic and infectious properties of the prion protein." J Virol **77**(13): 7611-22.

Cohen, F. E., K. M. Pan, et al. (1994). "Structural clues to prion replication." Science **264**(5158): 530-1.

Collee, J. G. and R. Bradley (1997). "BSE: a decade on--Part I." Lancet **349**(9052): 636-41.

Collinge, J. (1997). "Human prion diseases and bovine spongiform encephalopathy (BSE)." Hum Mol Genet **6**(10): 1699-705.

Collinge, J., J. Brown, et al. (1992). "Inherited prion disease with 144 base pair gene insertion. 2. Clinical and pathological features." Brain **115 (Pt 3)**: 687-710.

Collinge, J. and A. R. Clarke (2007). "A general model of prion strains and their pathogenicity." Science **318**(5852): 930-6.

Collinge, J., M. S. Palmer, et al. (1991). "Genetic predisposition to iatrogenic Creutzfeldt-Jakob disease." Lancet **337**(8755): 1441-2.

Collinge, J. and M. Rossor (1996). "A new variant of prion disease." Lancet **347**(9006): 916-7.

Collinge, J., K. C. Sidle, et al. (1996). "Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD." Nature **383**(6602): 685-90.

Collinge, J., M. A. Whittington, et al. (1994). "Prion protein is necessary for normal synaptic function." Nature **370**(6487): 295-7.

Creutzfeldt, H. G. (1920). "Ueber eine eigenartige herdförmige Erkrankung des Zentralnervensystem." Z. Gesamte Neurol. Psychiatrie(57): 1-18.

Cuillé, J., Chelle, P.L. (1936). "La maladie dite "tremblante" du mouton; est-elle inoculable?" Compte Rend Acad Sci **203**(1552).

DeArmond, S. J., W. C. Mobley, et al. (1987). "Changes in the localization of brain prion proteins during scrapie infection." Neurology **37**(8): 1271-80.

E. Flechsig, I. H., A. Aguzzi, C. Weissmann (unpublished results).

Ebenbichler, C. F., N. M. Thielens, et al. (1991). "Human immunodeficiency virus type 1 activates the classical pathway of complement by direct C1 binding through specific sites in the transmembrane glycoprotein gp41." J Exp Med **174**(6): 1417-24.

Endres, R., M. B. Alimzhanov, et al. (1999). "Mature follicular dendritic cell networks depend on expression of lymphotoxin beta receptor by radioresistant stromal cells and of lymphotoxin beta and tumor necrosis factor by B cells." J Exp Med **189**(1): 159-68.

Falsig, J. and A. Aguzzi (2008). "The prion organotypic slice culture assay--POSCA." Nat Protoc **3**(4): 555-62.

Fevrier, B., D. Vilette, et al. (2004). "Cells release prions in association with exosomes." Proc Natl Acad Sci U S A **101**(26): 9683-8.

Fischer, M., T. Rulicke, et al. (1996). "Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie." Embo J **15**(6): 1255-64.

Flechsig, E., I. Hegyi, et al. (2003). "Expression of truncated PrP targeted to Purkinje cells of PrP knockout mice causes Purkinje cell death and ataxia." Embo J **22**(12): 3095-101.

Flechsig, E., D. Shmerling, et al. (2000). "Prion protein devoid of the octapeptide repeat region restores susceptibility to scrapie in PrP knockout mice." Neuron **27**(2): 399-408.

Flechsig, E. and C. Weissmann (2004). "The role of PrP in health and disease." Curr Mol Med **4**(4): 337-53.

Ford, M. J., L. J. Burton, et al. (2002). "Selective expression of prion protein in peripheral tissues of the adult mouse." Neuroscience **113**(1): 177-92.

Forster, R., A. E. Mattis, et al. (1996). "A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen." Cell **87**(6): 1037-47.

Fu, Y. X., H. Molina, et al. (1997). "Lymphotoxin-alpha (LTalpha) supports development of splenic follicular structure that is required for IgG responses." J Exp Med **185**(12): 2111-20.

Gajdusek, D. C. (1977). "Unconventional viruses and the origin and disappearance of kuru." Science **197**(4307): 943-60.

Gajdusek, D. C., C. J. Gibbs, et al. (1966). "Experimental transmission of a Kuru-like syndrome to chimpanzees." Nature **209**(5025): 794-6.

Gajdusek, D. C. and V. Zigas (1957). "Degenerative disease of the central nervous system in New Guinea; the endemic occurrence of kuru in the native population." N Engl J Med **257**(20): 974-8.

Genoud, N., A. Behrens, et al. (2004). "Disruption of Doppel prevents neurodegeneration in mice with extensive Prnp deletions." Proc Natl Acad Sci U S A **101**(12): 4198-203.

Gerstmann, J. (1928). "Ueber ein noch nicht beschriebenes Reflexphanomen bei einer Erkrankung des zerebellaren Systems." Wien Med Wochenschr(78): 906-908.

Gibbs, C. J., Jr., D. C. Gajdusek, et al. (1968). "Creutzfeldt-Jakob disease (spongiform encephalopathy): transmission to the chimpanzee." Science **161**(839): 388-9.

Glatzel, M., E. Abela, et al. (2003). "Extraneural pathologic prion protein in sporadic Creutzfeldt-Jakob disease." N Engl J Med **349**(19): 1812-20.

Glatzel, M. and A. Aguzzi (2000). "PrP(C) expression in the peripheral nervous system is a determinant of prion neuroinvasion." J Gen Virol **81**(Pt 11): 2813-21.

Glatzel, M., F. L. Heppner, et al. (2001). "Sympathetic innervation of lymphoreticular organs is rate limiting for prion neuroinvasion." Neuron **31**(1): 25-34.

Goldfarb, L. G. (2002). "Kuru: the old epidemic in a new mirror." Microbes Infect **4**(8): 875-82.

Gommerman, J. L. and J. L. Browning (2003). "Lymphotoxin/light, lymphoid microenvironments and autoimmune disease." Nat Rev Immunol **3**(8): 642-55.

Gonzalez, M., F. Mackay, et al. (1998). "The sequential role of lymphotoxin and B cells in the development of splenic follicles." J Exp Med **187**(7): 997-1007.

Gordon, W. S. (1946). "Advances in veterinary research." Vet Rec **58**: 516-520.

Griffith, J. S. (1967). "Self-replication and scrapie." Nature **215**(5105): 1043-4.

Hadlow, W. J. (1995). "Neuropathology and the scrapie-kuru connection." Brain Pathol **5**(1): 27-31.

Halliwell, B. (2006). "Oxidative stress and neurodegeneration: where are we now?" J Neurochem **97**(6): 1634-58.

Haraguchi, T., S. Fisher, et al. (1989). "Asparagine-linked glycosylation of the scrapie and cellular prion proteins." Arch Biochem Biophys **274**(1): 1-13.

Harris, D. A., M. T. Huber, et al. (1993). "Processing of a cellular prion protein: identification of N- and C-terminal cleavage sites." Biochemistry **32**(4): 1009-16.

Harris, D. A., P. Lele, et al. (1993). "Localization of the mRNA for a chicken prion protein by in situ hybridization." Proc Natl Acad Sci U S A **90**(9): 4309-13.

Hartsough, G. R. and D. Burger (1965). "Encephalopathy of mink. I. Epizootiologic and clinical observations." J Infect Dis **115**(4): 387-92.

Hayward, P. A., J. E. Bell, et al. (1994). "Prion protein immunocytochemistry: reliable protocols for the investigation of Creutzfeldt-Jakob disease." Neuropathol Appl Neurobiol **20**(4): 375-83.

Heikenwalder, M., C. Julius, et al. (2007). "Prions and peripheral nerves: a deadly rendezvous." J Neurosci Res **85**(12): 2714-25.

Hill, A. F., M. Desbruslais, et al. (1997). "The same prion strain causes vCJD and BSE." Nature **389**(6650): 448-50, 526.

Hill, A. F., S. Joiner, et al. (2000). "Species-barrier-independent prion replication in apparently resistant species." Proc Natl Acad Sci U S A **97**(18): 10248-53.

Hooper, N. M. (2005). "Roles of proteolysis and lipid rafts in the processing of the amyloid precursor protein and prion protein." Biochem Soc Trans **33**(Pt 2): 335-8.

Hope, J., G. Multhaup, et al. (1988). "Molecular pathology of scrapie-associated fibril protein (PrP) in mouse brain affected by the ME7 strain of scrapie." Eur J Biochem **172**(2): 271-7.

Horn, G. (2001). review of the origin of BSE. London, Department for Environment, Food and Rural Affairs (DEFRA) Publications.

Huang, F. P., C. F. Farquhar, et al. (2002). "Migrating intestinal dendritic cells transport PrP(Sc) from the gut." J Gen Virol **83**(Pt 1): 267-71.

Huillard d'Aignaux, J., D. Costagliola, et al. (1999). "Incubation period of Creutzfeldt-Jakob disease in human growth hormone recipients in France." Neurology **53**(6): 1197-201.

Hutter, G., F. L. Heppner, et al. (2003). "No superoxide dismutase activity of cellular prion protein in vivo." Biol Chem **384**(9): 1279-85.

Jakob, A. (1921). "Ueber eigenartige Erkrankungen des Zentralnervensystem mit bemerkenswretem anatomischen Befunde." Z. Gesamte Neurol. Psychiatrie(64): 147-228.

Jeffrey, M., S. Martin, et al. (2001). "Onset of accumulation of PrPres in murine ME7 scrapie in relation to pathological and PrP immunohistochemical changes." J Comp Pathol **124**(1): 20-8.

Jendroska, K., F. P. Heinzel, et al. (1991). "Proteinase-resistant prion protein accumulation in Syrian hamster brain correlates with regional pathology and scrapie infectivity." Neurology **41**(9): 1482-90.

Jimenez-Huete, A., P. M. Lievens, et al. (1998). "Endogenous proteolytic cleavage of normal and disease-associated isoforms of the human prion protein in neural and non-neural tissues." Am J Pathol **153**(5): 1561-72.

Johnson, C. J., K. E. Phillips, et al. (2006). "Prions adhere to soil minerals and remain infectious." PLoS Pathog **2**(4): e32.

Jones, E. M. and W. K. Surewicz (2005). "Fibril conformation as the basis of species- and strain-dependent seeding specificity of mammalian prion amyloids." Cell **121**(1): 63-72.

Kaeser, P. S., M. A. Klein, et al. (2001). "Efficient lymphoreticular prion propagation requires PrP(c) in stromal and hematopoietic cells." J Virol **75**(15): 7097-106.

Kaneko, K., M. Vey, et al. (1997). "COOH-terminal sequence of the cellular prion protein directs subcellular trafficking and controls conversion into the scrapie isoform." Proc Natl Acad Sci U S A **94**(6): 2333-8.

Kincaid, A. E. and J. C. Bartz (2007). "The nasal cavity is a route for prion infection in hamsters." J Virol **81**(9): 4482-91.

Kitamoto, T., R. Iizuka, et al. (1993). "An amber mutation of prion protein in Gerstmann-Straussler syndrome with mutant PrP plaques." Biochem Biophys Res Commun **192**(2): 525-31.

Kitamura, D., J. Roes, et al. (1991). "A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene." Nature **350**(6317): 423-6.

Klatzo, I., D. C. Gajdusek, et al. (1959). "Pathology of Kuru." Lab Invest **8**(4): 799-847.

Klein, M. A., R. Frigg, et al. (1997). "A crucial role for B cells in neuroinvasive scrapie." Nature **390**(6661): 687-90.

Klein, M. A., P. S. Kaeser, et al. (2001). "Complement facilitates early prion pathogenesis." Nat Med **7**(4): 488-92.

Koni, P. A., R. Sacca, et al. (1997). "Distinct roles in lymphoid organogenesis for lymphotoxins alpha and beta revealed in lymphotoxin beta-deficient mice." Immunity **6**(4): 491-500.

Kuwahara, C., A. M. Takeuchi, et al. (1999). "Prions prevent neuronal cell-line death." Nature **400**(6741): 225-6.

Lampert, P. W., D. C. Gajdusek, et al. (1972). "Subacute spongiform virus encephalopathies. Scrapie, Kuru and Creutzfeldt-Jakob disease: a review." Am J Pathol **68**(3): 626-52.

Lasmezas, C. I., J. P. Deslys, et al. (1997). "Transmission of the BSE agent to mice in the absence of detectable abnormal prion protein." Science **275**(5298): 402-5.

Li, A., H. M. Christensen, et al. (2007). "Neonatal lethality in transgenic mice expressing prion protein with a deletion of residues 105-125." Embo J **26**(2): 548-58.

Liao, Y. C., R. V. Lebo, et al. (1986). "Human prion protein cDNA: molecular cloning, chromosomal mapping, and biological implications." Science **233**(4761): 364-7.

Linsell, L., S. N. Cousens, et al. (2004). "A case-control study of sporadic Creutzfeldt-Jakob disease in the United Kingdom: analysis of clustering." Neurology **63**(11): 2077-83.

Locht, C., B. Chesebro, et al. (1986). "Molecular cloning and complete sequence of prion protein cDNA from mouse brain infected with the scrapie agent." Proc Natl Acad Sci U S A **83**(17): 6372-6.

Lu, K., W. Wang, et al. (2000). "Expression and structural characterization of the recombinant human doppel protein." Biochemistry **39**(44): 13575-83.

Ma, J., R. Wollmann, et al. (2002). "Neurotoxicity and neurodegeneration when PrP accumulates in the cytosol." Science **298**(5599): 1781-5.

Mabbott, N. A., M. E. Bruce, et al. (2001). "Temporary depletion of complement component C3 or genetic deficiency of C1q significantly delays onset of scrapie." Nat Med **7**(4): 485-7.

Mabbott, N. A., F. Mackay, et al. (2000). "Temporary inactivation of follicular dendritic cells delays neuroinvasion of scrapie." Nat Med **6**(7): 719-20.

Mabbott, N. A., J. Young, et al. (2003). "Follicular dendritic cell dedifferentiation by treatment with an inhibitor of the lymphotoxin pathway dramatically reduces scrapie susceptibility." J Virol **77**(12): 6845-54.

Mackay, F. and J. L. Browning (1998). "Turning off follicular dendritic cells." Nature **395**(6697): 26-7.

Madec, J. Y., M. H. Groschup, et al. (2000). "Protease-resistant prion protein in brain and lymphoid organs of sheep within a naturally scrapie-infected flock." Microb Pathog **28**(6): 353-62.

Mallucci, G., A. Dickinson, et al. (2003). "Depleting neuronal PrP in prion infection prevents disease and reverses spongiosis." Science **302**(5646): 871-4.

Mallucci, G. R., S. Ratte, et al. (2002). "Post-natal knockout of prion protein alters hippocampal CA1 properties, but does not result in neurodegeneration." Embo J **21**(3): 202-10.

Mange, A., F. Beranger, et al. (2004). "Alpha- and beta- cleavages of the amino-terminus of the cellular prion protein." Biol Cell **96**(2): 125-32.

Manson, J., J. D. West, et al. (1992). "The prion protein gene: a role in mouse embryogenesis?" Development **115**(1): 117-22.

Manson, J. C., A. R. Clarke, et al. (1994). "129/Ola mice carrying a null mutation in PrP that abolishes mRNA production are developmentally normal." Mol Neurobiol **8**(2-3): 121-7.

Marella, M., S. Lehmann, et al. (2002). "Filipin prevents pathological prion protein accumulation by reducing endocytosis and inducing cellular PrP release." J Biol Chem **277**(28): 25457-64.

Marsh, R. F., R. A. Bessen, et al. (1991). "Epidemiological and experimental studies on a new incident of transmissible mink encephalopathy." J Gen Virol **72 (Pt 3)**: 589-94.

Marsh, R. F. and W. J. Hadlow (1992). "Transmissible mink encephalopathy." Rev Sci Tech **11**(2): 539-50.

Mastrangelo, P. and D. Westaway (2001). "Biology of the prion gene complex." Biochem Cell Biol **79**(5): 613-28.

Mastrangelo, P. and D. Westaway (2001). "The prion gene complex encoding PrP(C) and Doppel: insights from mutational analysis." Gene **275**(1): 1-18.

Mathiason, C. K., J. G. Powers, et al. (2006). "Infectious prions in the saliva and blood of deer with chronic wasting disease." Science **314**(5796): 133-6.

McKinley, M. P., R. K. Meyer, et al. (1991). "Scrapie prion rod formation in vitro requires both detergent extraction and limited proteolysis." J Virol **65**(3): 1340-51.

McLennan, N. F., P. M. Brennan, et al. (2004). "Prion protein accumulation and neuroprotection in hypoxic brain damage." Am J Pathol **165**(1): 227-35.

McMahon, H. E., A. Mange, et al. (2001). "Cleavage of the amino terminus of the prion protein by reactive oxygen species." J Biol Chem **276**(3): 2286-91.

Medori, R., H. J. Tritschler, et al. (1992). "Fatal familial insomnia, a prion disease with a mutation at codon 178 of the prion protein gene." N Engl J Med **326**(7): 444-9.

Meyer, R. K., M. P. McKinley, et al. (1986). "Separation and properties of cellular and scrapie prion proteins." Proc Natl Acad Sci U S A **83**(8): 2310-4.

Miele, G., A. R. Alejo Blanco, et al. (2003). "Embryonic activation and developmental expression of the murine prion protein gene." Gene Expr **11**(1): 1-12.

Mo, H., R. C. Moore, et al. (2001). "Two different neurodegenerative diseases caused by proteins with similar structures." Proc Natl Acad Sci U S A **98**(5): 2352-7.

Mohan, J., J. Hopkins, et al. (2005). "Skin-derived dendritic cells acquire and degrade the scrapie agent following in vitro exposure." Immunology **116**(1): 122-33.

Montrasio, F., R. Frigg, et al. (2000). "Impaired prion replication in spleens of mice lacking functional follicular dendritic cells." Science **288**(5469): 1257-9.

Moore, R. C., I. Y. Lee, et al. (1999). "Ataxia in prion protein (PrP)-deficient mice is associated with upregulation of the novel PrP-like protein doppel." J Mol Biol **292**(4): 797-817.

Moser, M., R. J. Colello, et al. (1995). "Developmental expression of the prion protein gene in glial cells." Neuron **14**(3): 509-17.

Mouillet-Richard, S., M. Ermonval, et al. (2000). "Signal transduction through prion protein." Science **289**(5486): 1925-8.

Muramoto, T., S. J. DeArmond, et al. (1997). "Heritable disorder resembling neuronal storage disease in mice expressing prion protein with deletion of an alpha-helix." Nat Med **3**(7): 750-5.

Naslavsky, N., H. Shmeeda, et al. (1999). "Sphingolipid depletion increases formation of the scrapie prion protein in neuroblastoma cells infected with prions." J Biol Chem **274**(30): 20763-71.

Naslavsky, N., R. Stein, et al. (1997). "Characterization of detergent-insoluble complexes containing the cellular prion protein and its scrapie isoform." J Biol Chem **272**(10): 6324-31.

Neary, K., B. Caughey, et al. (1991). "Protease sensitivity and nuclease resistance of the scrapie agent propagated in vitro in neuroblastoma cells." J Virol **65**(2): 1031-4.

Neuendorf, E., A. Weber, et al. (2004). "Glycosylation deficiency at either one of the two glycan attachment sites of cellular prion protein preserves susceptibility to bovine spongiform encephalopathy and scrapie infections." J Biol Chem **279**(51): 53306-16.

Nilsson, K. P., A. Herland, et al. (2005). "Conjugated polyelectrolytes: conformation-sensitive optical probes for detection of amyloid fibril formation." Biochemistry **44**(10): 3718-24.

Oesch, B., D. Westaway, et al. (1985). "A cellular gene encodes scrapie PrP 27-30 protein." Cell **40**(4): 735-46.

Paitel, E., C. Alves da Costa, et al. (2002). "Overexpression of PrPc triggers caspase 3 activation: potentiation by proteasome inhibitors and blockade by anti-PrP antibodies." J Neurochem **83**(5): 1208-14.

Palmer, M. S., A. J. Dryden, et al. (1991). "Homozygous prion protein genotype predisposes to sporadic Creutzfeldt-Jakob disease." Nature **352**(6333): 340-2.

Parchi, P., R. Castellani, et al. (1996). "Molecular basis of phenotypic variability in sporadic Creutzfeldt-Jakob disease." Ann Neurol **39**(6): 767-78.

Parchi, P., R. Castellani, et al. (1995). "Regional distribution of protease-resistant prion protein in fatal familial insomnia." Ann Neurol **38**(1): 21-9.

Pauly, P. C. and D. A. Harris (1998). "Copper stimulates endocytosis of the prion protein." J Biol Chem **273**(50): 33107-10.

Peoc'h, K., C. Guerin, et al. (2000). "First report of polymorphisms in the prion-like protein gene (PRND): implications for human prion diseases." Neurosci Lett **286**(2): 144-8.

Perera, W. S. and N. M. Hooper (2001). "Ablation of the metal ion-induced endocytosis of the prion protein by disease-associated mutation of the octarepeat region." Curr Biol **11**(7): 519-23.

Peretz, D., M. R. Scott, et al. (2001). "Strain-specified relative conformational stability of the scrapie prion protein." Protein Sci **10**(4): 854-63.

Peters, P. J., A. Mironov, Jr., et al. (2003). "Trafficking of prion proteins through a caveolae-mediated endosomal pathway." J Cell Biol **162**(4): 703-17.

Plummer, P. J. (1946). "Scrapie-A Disease of Sheep: A Review of the literature." Can J Comp Med Vet Sci **10**(2): 49-54.

Porto-Carreiro, I., B. Fevrier, et al. (2005). "Prions and exosomes: from PrPc trafficking to PrPsc propagation." Blood Cells Mol Dis **35**(2): 143-8.

Poser, C. M. (2002). "Notes on the history of the prion diseases. Part I." Clin Neurol Neurosurg **104**(1): 1-9.

Prinz, M., M. Heikenwalder, et al. (2003). "Positioning of follicular dendritic cells within the spleen controls prion neuroinvasion." Nature **425**(6961): 957-62.

Prinz, M., G. Huber, et al. (2003). "Oral prion infection requires normal numbers of Peyer's patches but not of enteric lymphocytes." Am J Pathol **162**(4): 1103-11.

Prinz, M., F. Montrasio, et al. (2002). "Lymph nodal prion replication and neuroinvasion in mice devoid of follicular dendritic cells." Proc Natl Acad Sci U S A **99**(2): 919-24.

Prusiner, S. (2004). Prion Biology and Disease. New York, Cold spring Harbor Laboratory Press.

Prusiner, S. B. (1982). "Novel proteinaceous infectious particles cause scrapie." Science **216**(4542): 136-44.

Prusiner, S. B. (1989). "Scrapie prions." Annu Rev Microbiol **43**: 345-74.

Prusiner, S. B., S. P. Cochran, et al. (1982). "Measurement of the scrapie agent using an incubation time interval assay." Ann Neurol **11**(4): 353-8.

Riek, R., S. Hornemann, et al. (1996). "NMR structure of the mouse prion protein domain PrP(121-321)." Nature **382**(6587): 180-2.

Riek, R., S. Hornemann, et al. (1997). "NMR characterization of the full-length recombinant murine prion protein, mPrP(23-231)." FEBS Lett **413**(2): 282-8.

Riek, R. and T. Luhrs (2003). "Three-dimensional structures of the prion protein and its doppel." Clin Lab Med **23**(1): 209-25.

Rivera-Milla, E., C. A. Stuermer, et al. (2003). "An evolutionary basis for scrapie disease: identification of a fish prion mRNA." Trends Genet **19**(2): 72-5.

Roos, R., D. C. Gajdusek, et al. (1973). "The clinical characteristics of transmissible Creutzfeldt-Jakob disease." Brain **96**(1): 1-20.

Rossi, D., A. Cozzio, et al. (2001). "Onset of ataxia and Purkinje cell loss in PrP null mice inversely correlated with Dpl level in brain." Embo J **20**(4): 694-702.

Roucou, X., P. N. Giannopoulos, et al. (2005). "Cellular prion protein inhibits proapoptotic Bax conformational change in human neurons and in breast carcinoma MCF-7 cells." Cell Death Differ **12**(7): 783-95.

Roucou, X., Q. Guo, et al. (2003). "Cytosolic prion protein is not toxic and protects against Bax-mediated cell death in human primary neurons." J Biol Chem **278**(42): 40877-81.

Safar, J., P. P. Roller, et al. (1993). "Conformational transitions, dissociation, and unfolding of scrapie amyloid (prion) protein." J Biol Chem **268**(27): 20276-84.

Safar, J. G., P. Lessard, et al. (2008). "Transmission and detection of prions in feces." J Infect Dis **198**(1): 81-9.

Sakaguchi, S., S. Katamine, et al. (1996). "Loss of cerebellar Purkinje cells in aged mice homozygous for a disrupted PrP gene." Nature **380**(6574): 528-31.

Sales, N., R. Hassig, et al. (2002). "Developmental expression of the cellular prion protein in elongating axons." Eur J Neurosci **15**(7): 1163-77.

Santuccione, A., V. Sytnyk, et al. (2005). "Prion protein recruits its neuronal receptor NCAM to lipid rafts to activate p59fyn and to enhance neurite outgrowth." J Cell Biol **169**(2): 341-54.

Sarnataro, D., V. Campana, et al. (2004). "PrP(C) association with lipid rafts in the early secretory pathway stabilizes its cellular conformation." Mol Biol Cell **15**(9): 4031-42.

Sarradet, M. (1883). "Un cas de tremblante sur un boeuf." Rev Vet **3**: 310-12.

Schneider, B., V. Mutel, et al. (2003). "NADPH oxidase and extracellular regulated kinases 1/2 are targets of prion protein signaling in neuronal and nonneuronal cells." Proc Natl Acad Sci U S A **100**(23): 13326-31.

Scott, J. R., J. D. Foster, et al. (1993). "Conjunctival instillation of scrapie in mice can produce disease." Vet Microbiol **34**(4): 305-9.

Scott, M., D. Foster, et al. (1989). "Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques." Cell **59**(5): 847-57.

Seeger, H., M. Heikenwalder, et al. (2005). "Coincident scrapie infection and nephritis lead to urinary prion excretion." Science **310**(5746): 324-6.

Sellier, P. (2003). "Protein nutrition for ruminants in European countries, in the light of animal feeding regulations linked to bovine spongiform encephalopathy." Rev Sci Tech **22**(1): 259-69.

Shmerling, D., I. Hegyi, et al. (1998). "Expression of amino-terminally truncated PrP in the mouse leading to ataxia and specific cerebellar lesions." Cell **93**(2): 203-14.

Shyng, S. L., J. E. Heuser, et al. (1994). "A glycolipid-anchored prion protein is endocytosed via clathrin-coated pits." J Cell Biol **125**(6): 1239-50.

Sigurdson, C. J. (2008). "A prion disease of cervids: Chronic wasting disease." Vet Res **39**(4): 41.

Sigurdson, C. J. and M. W. Miller (2003). "Other animal prion diseases." Br Med Bull **66**: 199-212.

Sigurdson, C. J., K. P. Nilsson, et al. (2007). "Prion strain discrimination using luminescent conjugated polymers." Nat Methods **4**(12): 1023-30.

Sigurdson, C. J., T. R. Spraker, et al. (2001). "PrP(CWD) in the myenteric plexus, vagosympathetic trunk and endocrine glands of deer with chronic wasting disease." J Gen Virol **82**(Pt 10): 2327-34.

Silverman, G. L., K. Qin, et al. (2000). "Doppel is an N-glycosylated, glycosylphosphatidylinositol-anchored protein. Expression in testis and ectopic production in the brains of Prnp(0/0) mice predisposed to Purkinje cell loss." J Biol Chem **275**(35): 26834-41.

Smith, P. G. and R. Bradley (2003). "Bovine spongiform encephalopathy (BSE) and its epidemiology." Br Med Bull **66**: 185-98.

Solforosi, L., J. R. Criado, et al. (2004). "Cross-linking cellular prion protein triggers neuronal apoptosis in vivo." Science **303**(5663): 1514-6.

Stahl, N., D. R. Borchelt, et al. (1987). "Scrapie prion protein contains a phosphatidylinositol glycolipid." Cell **51**(2): 229-40.

Sunyach, C., A. Jen, et al. (2003). "The mechanism of internalization of glycosylphosphatidylinositol-anchored prion protein." Embo J **22**(14): 3591-601.

Taraboulos, A., K. Jendroska, et al. (1992). "Regional mapping of prion proteins in brain." Proc Natl Acad Sci U S A **89**(16): 7620-4.

Taraboulos, A., M. Scott, et al. (1995). "Cholesterol depletion and modification of COOH-terminal targeting sequence of the prion protein inhibit formation of the scrapie isoform." J Cell Biol **129**(1): 121-32.

Tateishi, J., P. Brown, et al. (1995). "First experimental transmission of fatal familial insomnia." Nature **376**(6539): 434-5.

Taylor, D. M., I. McConnell, et al. (1996). "Scrapie infection can be established readily through skin scarification in immunocompetent but not immunodeficient mice." J Gen Virol **77 (Pt 7)**: 1595-9.

Taylor, D. R., N. T. Watt, et al. (2005). "Assigning functions to distinct regions of the N-terminus of the prion protein that are involved in its copper-stimulated, clathrin-dependent endocytosis." J Cell Sci **118**(Pt 21): 5141-53.

Tobler, I., S. E. Gaus, et al. (1996). "Altered circadian activity rhythms and sleep in mice devoid of prion protein." Nature **380**(6575): 639-42.

Tranulis, M. A., A. Espenes, et al. (2001). "The PrP-like protein Doppel gene in sheep and cattle: cDNA sequence and expression." Mamm Genome **12**(5): 376-9.

Tsui-Pierchala, B. A., M. Encinas, et al. (2002). "Lipid rafts in neuronal signaling and function." Trends Neurosci **25**(8): 412-7.

Tuzi, N. L., E. Gall, et al. (2002). "Expression of doppel in the CNS of mice does not modulate transmissible spongiform encephalopathy disease." J Gen Virol **83**(Pt 3): 705-11.

Vey, M., S. Pilkuhn, et al. (1996). "Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains." Proc Natl Acad Sci U S A **93**(25): 14945-9.

Wadsworth, J. D., A. F. Hill, et al. (2003). "Molecular and clinical classification of human prion disease." Br Med Bull **66**: 241-54.

Wadsworth, J. D., S. Joiner, et al. (2001). "Tissue distribution of protease resistant prion protein in variant Creutzfeldt-Jakob disease using a highly sensitive immunoblotting assay." Lancet **358**(9277): 171-80.

Waggoner, D. J., B. Drisaldi, et al. (2000). "Brain copper content and cuproenzyme activity do not vary with prion protein expression level." J Biol Chem **275**(11): 7455-8.

Ware, C. F., T. L. VanArsdale, et al. (1995). "The ligands and receptors of the lymphotoxin system." Curr Top Microbiol Immunol **198**: 175-218.

Watts, J. C. and D. Westaway (2007). "The prion protein family: diversity, rivalry, and dysfunction." Biochim Biophys Acta **1772**(6): 654-72.

Weissmann, C. (1991). "A 'unified theory' of prion propagation." Nature **352**(6337): 679-83.

Weissmann, C. (1999). "Molecular genetics of transmissible spongiform encephalopathies." J Biol Chem **274**(1): 3-6.

Weissmann, C. and A. Aguzzi (1997). "Bovine spongiform encephalopathy and early onset variant Creutzfeldt-Jakob disease." Curr Opin Neurobiol **7**(5): 695-700.

Weissmann, C. and A. Aguzzi (1999). "Perspectives: neurobiology. PrP's double causes trouble." Science **286**(5441): 914-5.

Weissmann, C., A. J. Raeber, et al. (2001). "Prions and the lymphoreticular system." Philos Trans R Soc Lond B Biol Sci **356**(1406): 177-84.

Wells, G. A., S. A. Hawkins, et al. (1998). "Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy (BSE): an update." Vet Rec **142**(5): 103-6.

Westaway, D. and G. A. Carlson (2002). "Mammalian prion proteins: enigma, variation and vaccination." Trends Biochem Sci **27**(6): 301-7.

Wickner, R. B., H. K. Edskes, et al. (2007). "Prions of fungi: inherited structures and biological roles." Nat Rev Microbiol **5**(8): 611-8.

Will, R. G., J. W. Ironside, et al. (1996). "A new variant of Creutzfeldt-Jakob disease in the UK." Lancet **347**(9006): 921-5.

Williams, A., P. J. Lucassen, et al. (1997). "PrP deposition, microglial activation, and neuronal apoptosis in murine scrapie." Exp Neurol **144**(2): 433-8.

Williams, E. S. and S. Young (1980). "Chronic wasting disease of captive mule deer: a spongiform encephalopathy." J Wildl Dis **16**(1): 89-98.

Williams, E. S. and S. Young (1992). "Spongiform encephalopathies in Cervidae." Rev Sci Tech **11**(2): 551-67.

Windl, O., M. Dempster, et al. (1995). "A candidate marsupial PrP gene reveals two domains conserved in mammalian PrP proteins." Gene **159**(2): 181-6.

Yoshikawa, D., N. Yamaguchi, et al. (2008). "Dominant-negative effects of the amino-terminal half of prion protein on neurotoxicity of PrP-like protein/doppel in mice." J Biol Chem.

Zabel, M. D., M. Heikenwalder, et al. (2007). "Stromal complement receptor CD21/35 facilitates lymphoid prion colonization and pathogenesis." J Immunol **179**(9): 6144-52.

Zanusso, G., S. Ferrari, et al. (2003). "Detection of pathologic prion protein in the olfactory epithelium in sporadic Creutzfeldt-Jakob disease." N Engl J Med **348**(8): 711-9.

VII. Acknowledgements

I thank all the people involved in this work.
The members of my PhD committee Adriano Aguzzi, Roger Morris and Peter Seeberger.
Sei-ichi Yusa, with whom I started working on the first project.
Thomas Rülicke and his laboratory for performing the pronuclei microinjections.
Mirzet Delic for taking care of my mice in the animal facility.
Li-Chun Infanger and Dimitri Goriounov for the genotyping of mice.
José Barros, Sophorn Chip, Jan Kranich, Nike Kraütler and Magdalini Polymenidou for daily help with technical issues.
Frank Baumann for help with the Southern blotting and many discussions, Jeppe Falsig and Anna-Maria Callela for the pulse-chase experiment.
Petra Schwarz and Rita Moos for maintaining the lab in a functional state.

VIII. Curriculum vitae

Personal information

Name	Claire Bridel
Date of birth	28.04.1978
Email	Claire.Bridel@usz.ch
Nationality	Swiss

Education

Since April 2005	MD-PhD student at the Institute for Neuropathology, University Hospital, Zurich
October 2004-April 2005	Molecular biology/biochemistry/genetics and statistics at the University of Zurich
April 2004-October 2004	Resident at the department of Neurosurgery, University Hospital, Lausanne
October 1997-January 2004	Medical studies at the University of Geneva
August 1993-June 1996	High school, Nyon Scientific A-levels (mathematics, physics, chemistry)